Isolation and Characterization of Bacteriophages for Bicontrol of *Salmonella* and Shiga Toxin-Producing *Escherichia coli* in Food Applications

A Thesis

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Dedication

This thesis is dedicated to my family: my parents, grandparents, aunts, uncles, and my brother. I can't express how much it means to me when you all tell me you're proud of me. No matter where we are or how far we have to travel to see one another, I always feel that I am home when I see you. I love you.

Abstract

Salmonella poses a significant risk to public health, with tens of thousands of cases occurring each year. Food is the primary vehicle for *Salmonella* outbreaks, and several diverse foods are frequently attributed to outbreaks. Traditional methods of pathogen control in the food industry are often indiscriminate, killing microbes that may be beneficial alongside the pathogens. In addition, these methods can alter the organoleptic properties of foods and may not be usable for raw and ready-to-eat foods such as raw poultry or fresh produce. Use of chemical antimicrobials is also growing out of favor in some settings as concerns rise over antimicrobial resistance in foodborne pathogens. Interest is growing in using phage cocktails as an alternative method to combat *Salmonella* and other foodborne pathogens.

Bacteriophages, or phages, are viruses that infect bacteria. They are highly host specific, safe to consume, relatively inexpensive, and do not alter the organoleptic properties of food, making them ideal as a biocontrol agent in a variety of food applications. Using several phages combined in a cocktail can increase their success in killing pathogens and lower the chance of resistance to the phages developing. Phages are the most abundant biological entity on the planet, and most remain undiscovered. A few commercial phage cocktails exist that may be used in the food industry, but identifying novel cocktails of unique phages increases the diversity of the tools available to handle troublesome pathogens that arise.

In this study, phages were isolated from local Minnesota wastewater samples. The newly isolated phages were tested for their ability to lyse and kill several serotypes of *Salmonella* and a few serotypes of Shiga-toxin producing *E. coli.* Six promising phages were picked for a putative novel cocktail. This putative cocktail was assessed for its ability to reduce *Salmonella* levels in a raw chicken breast model. The cocktail shows promise as a tool to manage both *Salmonella* and Shiga-toxin producing *E. coli* in food and food processing environments.

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1.1 *Salmonella*

1.1.1 Overview of Salmonella

Salmonella is a Gram-negative, rod-shaped bacteria belonging to the *Enterobacteriaceae* family and is made up of two species: *Salmonella bongori* and *Salmonella enterica.* *Salmonella enterica* is consists of six subspecies: *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *salamae* (II), *indica*(VI), and *enterica* (I) (Brenner et al., 2000). *Salmonella enterica* subsp*. enterica,* hereafter referred to generally as *Salmonella* is well-known for being the causative agent of foodborne salmonellosis. *Salmonella* is categorized into subgroups known as serotypes, which are characterized by differences in the antigenic structures: lipopolysaccharide (LPS), flagella, and capsular polysaccharide for a few serotypes – such as *Salmonella* Typhi. Over 2500 serotypes of *Salmonella* are recognized to date (Alikhan et al., 2018; Boyd et al., 1996; Brenner et al., 2000; Grimont & Weill, 2007; Popoff et al., 2004). However, only a few of these serotypes are frequently associated with human disease. Jones et al. (2008) analyzed over 46,000 cases of salmonellosis from 1996 to 2006, identifying just 687 serotypes that were implicated. From 1996 to 2011, 1280 serotypes of *Salmonella* were reported from human isolates, but just 864 of these appeared more than once (Boore et al., 2015). Additionally, salmonellosis outcome varied with serotype (Jones et al., 2008).

1.1.2 Salmonellosis

Salmonellosis is characterized as gastroenteritis with accompanying symptoms of diarrhea, abdominal pain and cramping, nausea, vomiting and headaches. Symptoms of salmonellosis frequently manifest in a range from six hours to twelve days after infection (Centers for Disease Control and Prevention [CDC], 2021a). The infections are generally self-limiting, with most symptoms subsiding in ten days or less (Eng et al., 2015). In severe cases, invasive *Salmonella* infections can occur, often manifesting as meningitis, bacteremia, osteomyelitis, or septic arthritis. Those most commonly impacted by invasive infections are children under the age of five, the elderly, and the immunocompromised. While most common symptoms of *Salmonella* subside on their own, some will continue to have long-lasting effects such as reactive arthritis, which can remain for months to years after infection (CDC, 2021a).

In the United States alone, *Salmonella* causes approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths each year. The primary vehicle for *Salmonella* outbreaks is contaminated food (CDC, 2021a), and the prevalence of *Salmonella* varies with geography and commodity (Boore et al., 2015; Ferrari et al., 2019; Jackson et al., 2013; Scallan et al., 2011; Worley et al., 2018). In the United States, the top five serotypes of *Salmonella* reported between 1996 and 2011 were Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana. In addition, overall rates of *Salmonella* infections in the United States have remained relatively constant, despite incidence rates between serotypes varying (Boore et al., 2015). *Salmonella* outbreaks are costly; they result in an economic burden of approximately $3.7 billion in medical costs annually in the United States (Hoffman et al., 2015). This does not include the costs to the food industry from foodborne *Salmonella*. In general, food safety incidents cause an estimated $7 billion loss in the U.S. economy each year (Hussain & Dawson, 2013). In one such *Salmonella* incident, an outbreak traced to peanut butter in 2007 resulted in an economic loss of $133 million. A 2009 outbreak associated with tomatoes cost the industry $250 million (Hussain & Dawson, 2013).

1.1.3 Foodborne Salmonella

Most *Salmonella* outbreaks in the U.S. are attributed to foods. While many major foodborne pathogens are primarily associated with one or two food commodities, *Salmonella* is uniquely associated with a wider range. Approximately 75% of *Salmonella* outbreaks can be attributed to the following seven food commodities: chicken, seeded vegetables, pork, fruits, other produce, eggs, and turkey (Interagency Food Safety Analytics Collaboration, 2020). *Salmonella* serotypes producing outbreaks vary with food commodity. From 1998 to 2008, most egg-associated *Salmonella* outbreaks were caused by serotypes Enteritidis and Heidelberg. For chicken, turkey, pork, and beef, the most attributed serotypes were Typhimurium, Enteritidis, Infantis, Newport, and Heidelberg. For leafy vegetables, the most commonly attributed serotypes were Newport and Javiana, while vine-stalk vegetables were primarily associated with Newport, Braenderup, Enteritidis, Javiana, and Typhimurium (Jackson et al., 2013). This variation is assumed to be a result of the different reservoirs each serotype typically resides in. Serotype-food associations are important to help hypothesize and identify sources of

outbreaks and contamination (Jackson et al., 2013). As with overall *Salmonella* illnesses, the rate of *Salmonella* infections attributed to food sources did not change between 2016 and 2019, despite variations in incidence of each serotype (Tock et al., 2019). Foodborne *Salmonella* remains a significant food safety concern to be addressed.

1.2 Shiga Toxin-Producing *Escherichia coli* (STEC)

1.2.1 Overview of STEC

Escherichia coli are Gram-negative, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. Most *E. coli* are harmless and are some of the organisms that make up the human gut microflora. However, Shiga toxin-producing *E. coli* (STEC) are strains of *E. coli* that have acquired genes for producing Shiga toxin. Like *Salmonella*, *E. coli* is classified into serotypes based on its antigens, O, H, and K. The O antigen is LPS, the H antigen is flagella, and the K antigen consists of capsular polysaccharides. STEC serotype O157:H7 is most commonly recognized, but over 200 serotypes of *E. coli* can produce Shiga toxin (Kim et al., 2020). Aside from O157:H7, six other serotypes make up over 75% of STEC infections in the United States: O26, O111, O103, O121, O45, and O145 (Minnesota Department of Health, 2019).

1.2.2 STEC infections

STEC is estimated to cause over 265,000 illnesses, 3,600 hospitalizations, and 30 deaths each year in the United States (Scallan et al., 2011). STEC is primarily spread through contaminated food or water, contact with an infected person or animal host. STEC infections frequently involve symptoms of diarrhea (which may be bloody), cramps, and vomiting. Symptoms can develop anywhere between one to ten days after exposure to STEC, but most commonly develop in three to four days (CDC, 2021b). Approximately 5-10% of those with a STEC infection will develop Hemolytic Uremic Syndrome (HUS), which may result in renal failure and death (CDC, 2021b). Those most susceptible to STEC are children under the age of five, the immunocompromised, and the elderly. Importantly, the infectious dose is very low, often even fewer than 10 cells (Etcheverría & Padola, 2013). From 1996 to 2016, overall rates of STEC infections have increased, with non-O157:H7 STEC driving the increase as O157:H7 rates have decreased since the peak in 2000 (CDC, 2018). Medical costs attributed to O157:H7 STEC and non-O157:H7 STEC outbreaks are estimated at $271 million and $27 million respectively (Hoffman et

al., 2015). As with *Salmonella*, the cost of STEC outbreaks from a food source are costly to the food industry is large as well. One outbreak of *E. coli* O157:H7 in spinach cost the industry $350 million in 2006 (Hussain & Dawson, 2013).

1.2.3 Foodborne STEC

Animals are the most common source of STEC transmission to humans and into the food system (J.-S. Kim et al., 2020). For STEC, row vegetables are the most common vehicle for foodborne outbreaks followed by beef (Interagency Food Safety Analytics Collaboration, 2020; Pires et al., 2011). Cattle serve as important reservoirs for STEC (Etcheverría & Padola, 2013), so the processing of beef or contamination of vegetable fields likely explain the attribution of STEC to beef and row vegetables. With STEC rates rising and low infectious dose, reducing STEC transmission through common food sources such as fresh produce and beef is a necessary public health measure.

1.3 *Salmonella* and STEC Control Methods in Food Systems

1.3.1 Methods

Pathogen control in foods is executed by a variety of measures, including irradiation, pasteurization , high pressure processing , cold atmospheric plasma, retort, freezing or chilling, pulsed electric field, and chemical antimicrobials (Amit et al., 2017; Beuchat & Ryu, 1997; Brito et al., 2002; Dhokane et al., 2006; Fernández et al., 2013). In fresh fruits and vegetables, pathogens can be internalized, so treatments including chlorine dioxide, electrolyzed water, UV light, cold atmospheric plasma, hydrogen peroxide, organic acids, acidified sodium chlorite, or high oxygen atmosphere with irradiation have shown promise in reducing pathogens both on the surface and internally (Olaimat & Holley, 2012). In addition to previously listed methods, hydrostatic pressure processing, active packaging, and natural antimicrobials have been employed in meat products (Sohaib et al., 2016). During slaughter, reducing pathogen levels on the carcasses is attempted using steam, water, and chemical antimicrobial solutions, and carcasses may be decontaminated by these methods prior to further processing (Buncic & Sofos, 2012). Despite numerous approaches for controlling pathogens, foodborne illness remains a significant concern in the United States. Additionally, each method has incompatibilities with certain food products, and several criticisms over these methods.

1.3.2 Criticisms

Several of the above methods utilize thermal means to reduce pathogens such as *Salmonella* and STEC, which is not always effective or compatible with preventing foodborne illness in certain products. Chilling and freezing prevent *Salmonella* and STEC growth, but do not kill the cells already contaminating the product. Thermal methods involving heat, such as pasteurization and retort, alter the organoleptic properties of the foods and may result in nutrient degradation (Amit et al., 2017). Physical means such as high pressure processing, irradiation, and drying also alter the organoleptic properties of foods (Moye et al., 2018). Irradiation in particular is unpopular with many consumers and known to accelerate lipid peroxidation (Brito et al., 2002). The presence of the radura label on foods causes mixed consumer reactions (Maherani et al., 2016), making it an unpopular labeling requirement for industry. Natural antimicrobials such as rosemary extract or garlic extract have proven effective in poultry applications and are clean label and GRAS, but they may impart off-flavors and odors that may not be acceptable to consumers (Grant & Parveen, 2017). Chemical antimicrobials and sanitizers are criticized for being harmful to the environment, becoming increasingly unpopular with consumers, and over concerns about resistant pathogens emerging (Moye et al., 2018).

A similarity between these methods is that they are indiscriminate (Moye et al., 2018) i.e., they do not kill only organisms of interest and interfere with the natural microflora of foods. Additionally, *Salmonella* and STEC are associated with meat, poultry, and fresh fruits and vegetables. These items are frequently handled and/or consumed raw by consumers, and physical or thermal processing methods for eliminating pathogens are not employed. In response to many of these concerns, interest in using bacteriophages as an alternative method for targeted control of *Salmonella* and STEC has increased, especially for raw and ready-to-eat (RTE) foods.

1.4 Bacteriophages

1.4.1 Overview of phages

Bacteriophages (phages) are viruses that infect bacteria. Phages were first discovered by Felix d'Herelle in 1916 and were named for their ability to "devour" bacteria (Sulakvelidze et al., 2001). There are over 10^{31} phage virions on Earth, making phages the most abundant entities on the planet (Akhtar et al., 2014; Bergh et al., 1989; Brüssow

& Hendrix, 2002; Cobián Güemes et al., 2016; Hatfull, 2008a). The majority of phages remain undiscovered (Cobián Güemes et al., 2016), though this is unsurprising due to their vast numbers. Many orders and families of phage have been identified, but this review will focus on phages of the order *Caudovirales*, which are tailed phages. This order encompasses the majority of phages studied at to date (Dion et al., 2020). *Caudovirales* phages are characterized by a polyhedral protein-based capsid, containing their double-stranded DNA, attached to a proteinaceous tail. The structures that a phage uses to interact with the host extend from the tail. Currently, five families make up *Caudovirales*:, *Myoviridae, Herelleviridae, Ackermannviridae, Siphoviridae and Podoviridae* (Dion et al., 2020). Phages in this order are classified into their families based on tail shape, length, and contractility, as well as their adsorption structures. *Myoviridae* and *Herelleviridae* share similar morphologies and are characterized by a polyhedral head, a long, inflexible, contractile tail with a protein sheath and tail fibers at the base of the tail. *Ackermannviridae* have head and tail structure similar to *Myoviridae* and *Herelleviridae* but have a unique and complex adsorption structure at the base of tail in place of the tail fibers. *Siphoviridae* have a polyhedral head, and a long, flexible, non-contractile tail without a sheath, along with tail fibers around the base of the tail. *Podoviridae* are defined by a polyhedral head, a short, non-contractile tail, and tail fibers at the base.

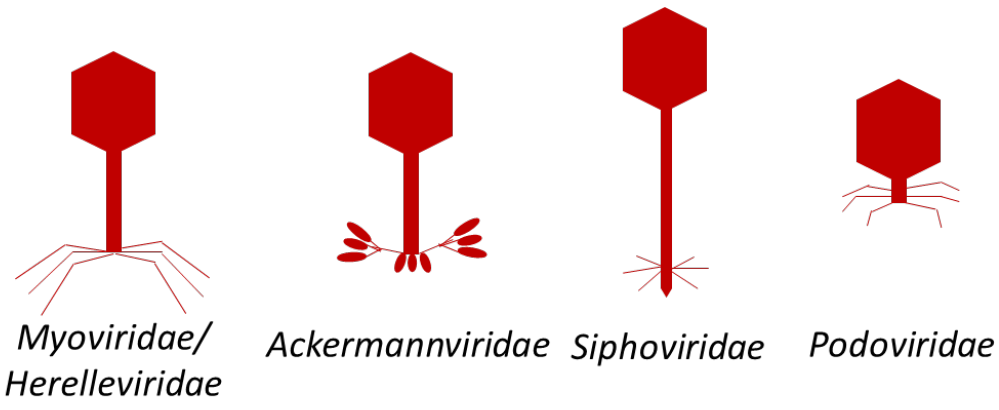


Figure 1.1 Phage family morphologies. Adapted from (Dion et al., 2020).

1.4.2 Phage lifecycles

Phages typically undergo one of two lifecycles, the lytic cycle or the lysogenic cycle – though other lifecycles, such as a pseudolysogenic lifecycle, have been identified (Cenens et al., 2013; Ripp & Miller, 1998). In both the lytic and lysogenic cycles, the phage adsorbs to its host receptor. Following this, an irreversible binding step occurs, and the phage will inject its DNA into the host cell. In the lytic cycle, the phage hijacks the host's replication machinery, utilizing it to produce progeny phages. The cycle ends with lysis and death of the host cell and release of progeny phage into the environment. These phages may then adsorb to a nearby host and begin the lytic cycle again. In contrast to the lytic cycle, the DNA of a phage undergoing the lysogenic cycle will be integrated into the host cell genome, where it will remain for an indeterminate amount of time. The integrated phage, known as a prophage, can later undergo the lytic cycle. Virulent phages are strictly lytic and cannot enter the lysogenic cycle, whereas temperate phages can enter either cycle, though they more commonly participate in the lysogenic cycle. During the lytic cycle, phages may mistakenly incorporate host DNA into a capsid. Such phages are known as transducing phages. When these phages bind to another host, they will inject DNA from the previous host into the cell. The new host cell may integrate this DNA into its genome. This process is known as generalized transduction, which can be performed by either temperate or virulent phages. In specialized transduction, genes nearby the phage genome, which has been incorporated to the host genome, may be mistakenly excised with the phage genome as it separates from the host genome. The improperly excised host DNA is then mistakenly incorporated into a capsid (Griffiths et al., 2000; Monteiro et al., 2019). The specialized transduction process can occur frequently with temperate phages, sometimes transferring DNA containing virulence factors or antimicrobial resistance factors from one host into another host, which may integrate that DNA into its genome. (Figueroa-Bossi et al., 2001; Monteiro et al., 2019). In lysogenic conversion, a phage with a genome containing a gene from a different host undergoes the lysogenic cycle, and the phage genome (containing a gene previously found in another host) is incorporated into the new host. Perhaps the most notorious example of this is the lysogenic conversion of non-pathogenic *E. coli* to Shiga-toxin producing *E. coli* by a temperate phage carrying genes encoding for Shiga-toxin production (Khalil et al., 2016;

Monteiro et al., 2019; Shaikh & Tarr, 2003). Concerns over lysogenic conversion by temperate phages has led to calls for phages being used in the food and agriculture sectors to be strictly lytic (Harrison & Brockhurst, 2017; Oliveira et al., 2015; Pirnay et al., 2015).

1.4.3 Host ranges and receptors

Phages are highly host specific. A phage that is effective in infecting one serotype of *Salmonella* may not infect another serotype of *Salmonella*. Sometimes a phage may even successfully infect one strain of *Salmonella* but not another strain of the same serotype. Some phages have been discovered that infect some serotypes of both *Salmonella* and *E. coli* (Bai et al., 2019a; Park et al., 2012). The host range of a phage is determined by a variety of factors, including the receptor it recognizes, host defense mechanisms, and the phage's ability to evade host defense mechanisms, among others.

A comprehensive review of host cell defense mechanisms can be found from de Jonge, et al. (2019), but, briefly, bacteria can evade infection through a variety of mechanisms, both extracellular and intracellular. The cell can sterically hinder the phage's ability to reach the receptor on the surface (Ernst et al., 2001; Guo et al., 1997; Harvey et al., 2018), induce capsular changes that hinder the phage (Fernandes & São-José, 2018; Scholl et al., 2005), or use outer membrane vesicles as decoys for the phage to adsorb to (Schwechheimer & Kuehn, 2015). Host cells can attempt to block phage DNA injection into the cell (L. R. García & Molineux, 1995). Once phage DNA enters the cell, additional defense mechanisms may be engaged to prevent replication and lysis, such as restriction modification systems that damage phage DNA (Loenen, 2003; Tock & Dryden, 2005), CRISPR systems (Barrangou et al., 2007; Makarova et al., 2015), abortive infection systems (Adams, 1955; Emond et al., 1997), BREX (Goldfarb et al., 2015), and DISARM (Ofir et al., 2018). Host cells may also suppress production of endolysins, which are enzymes used to lyse the cells to free the progeny phage (Roces et al., 2016). In addition to the many known phage defense system, it is very likely that many yet-uncharacterized systems exist for host cells to evade phage infection (Doron et al., 2018).

Despite the numerous anti-phage defenses employed, some phages have mechanisms to evade these defenses, as outlined in a review by (Samson et al., 2013a). For example, phages may be able to produce endonuclease enzyme inhibitors to block restriction modification systems from destroying its DNA (Rifat et al., 2008). Bacteria must also maneuver the balance between the cost of carrying defense systems in its genome and the benefit of evading phage with that system (van Houte et al., 2016). Moreover, any changes made for the purpose of evading one phage could instead make the host susceptible to another phage (Avrani et al., 2012; Ford et al., 2014).

Mutations in host receptors are among the most common method of evading phages (Mahichi et al., 2009). Adsorption is the first step in phage infection, and phages must be able to access and adsorb to a specific receptor on the host. Nearly any structure on the surface of the host cell can serve as a receptor (Bertozzi Silva et al., 2016), including antigens such as LPS and the flagella. However, modifications in surface structures could decrease host fitness, such as through loss of motility in an altered flagellar structure (Bai et al., 2019a) or loss of virulence.

Host receptors are major determinants in phage host ranges. Even a small change in a gene encoding a phage's receptor binding protein can alter the host range of the phage (Viana et al., 2015). Despite the importance of receptors for phage efficacy, they are often uncharacterized (Bai et al., 2019a; Gao et al., 2020; Islam et al., 2019; Petsong et al., 2019; Zschach et al., 2015). Common receptors for *Salmonella* phages, for those that have been assessed, are LPS, flagella, and porins such as OmpC and BtuB (Bai et al., 2019a; Lindberg & Holme, 1969; Marti et al., 2013). Some phages can recognize more than one receptor, such as phage SP6 which has two types of receptor binding proteins to increase its host range (Golomidova et al., 2016; Tu et al., 2017). Optimal phages would target receptors that are virulence factors so that phage-resistant mutants may have been forced to reduce their virulence in the process of becoming resistant (Kortright et al., 2019). Overall, understanding phage receptors and other determinants of phage host ranges allows for a targeted approach to controlling specific *Salmonella* and STEC serotypes and strains that are proving problematic in the food industry.

1.5 Bacteriophages for Biocontrol in the Food System

1.5.1 Benefits

Phage use has been proposed for controlling *Salmonella* and STEC at nearly every point along the production process, from pre-harvest (in both fresh produce and in livestock) to finished product packaging and processing environment sanitation (Sulakvelidze, 2013). The host specificity of phages mean that non-target organisms will not be affected, which is especially important in fermentation processes. Because phages occur naturally in foods (Atterbury et al., 2003a, 2003b; Bao et al., 2015; Brüssow & Hendrix, 2002; Gautier et al., 1995; Hsu et al., 2002; Hudson et al., 2005; Kennedy et al., 1984; Sillankorva et al., 2012), they will not alter the normal microflora of foods (Moye et al., 2018; O'Sullivan et al., 2019). Phages are also abundant in the human gut (Dalmasso et al., 2014). This, combined with the specificity of phages, indicates that they will not interfere with the gut microbiome when consumed. Furthermore, ingestion studies of phages have indicated that they are safe for consumption, with no signs of phages causing deleterious effects to those who consumed them (Alisky et al., 1998; Bruttin & Brüssow, 2005; McCallin et al., 2013a). Phages are relatively inexpensive to produce (Loc-Carrillo & Abedon, 2011; Nilsson, 2014): they are propagated in large cultures of their host, and the major cost of purifying phages away from any hazardous components of the host is decreasing (Loc-Carrillo & Abedon, 2011). Because phages are so ubiquitous, they are considered natural and may be utilized in organic products (Moye et al., 2018), which have grown greatly in popularity in recent years (Reganold & Wachter, 2016). Consumer acceptance studies have shown that consumers would be willing to pay more for phage-treated produce per pound when informed about the ability of phages to increase food safety (Naanwaab et al., 2014). Lastly, analysis of phage-treated foods indicated that the organoleptic properties are not affected by phage application (Otto et al., 2011; Perera et al., 2015; Pietracha & Misiewicz, 2016). Phages have many beneficial components that make them an optimal biocontrol strategy in the food industry that are likely to be accepted by consumers due to their safety, natural presence, and the preservation of organoleptic properties of foods. Additionally, for industry phages are relatively inexpensive and unlikely to impact off-target organisms. These qualities appear to make

phages an optimal solution to food safety problems from both the industry and consumer standpoint. However, bacteriophage biocontrol strategies are not without criticism.

1.5.2 Criticisms

The food industry has mixed opinions over utilizing phage as a primary biocontrol strategy, especially because some phages pose threats to fermentations (de Melo et al., 2018). In addition, phages oftentimes can survive a wider range of conditions and sanitation treatments than their hosts and can remain in the processing environment (Fister et al., 2016; Ly-Chatain, 2014; Meaden & Koskella, 2013), causing concern over false positive results during pathogen testing (Brown-Jaque et al., 2016; Muniesa et al., 2005). Perhaps the most significant concern is over pathogens developing resistance to phages (Andreatti Filho et al., 2007; Cairns & Payne, 2008; Carey-Smith et al., 2006; Greer, 2005; Y. Hong et al., 2016). This is inevitable due to evolutionary pressures and constant co-evolution of phages and their hosts (Lenski, 1984). However, phage resistance is considered easier to overcome than small molecule antimicrobial resistance (Capparelli et al., 2007; Labrie et al., 2010). Phages are often utilized in foods that are kept at refrigerated temperatures where many pathogens will not grow. This means that phage replication will not occur during storage, so resistance should not arise in any significant capacity (Hudson et al., 2005). At least one study indicated that presence of resistant cells did not hinder the phage efficacy in their food challenge model (O'Flynn et al., 2004). Still, to avoid resistance a cocktail of phages with different receptors is advised for biocontrol of pathogens in foods (Andreatti Filho et al., 2007; Bai et al., 2019; Chan et al., 2013; Goodridge & Bisha, 2011; Hudson et al., 2005; Jason & Paul, 2009; M. Kim & Ryu, 2012; Kortright et al., 2019).

1.5.3 Applications

A small number of commercial cocktails, mixtures of more than one phage, are available for *Salmonella* and STEC biocontrol in the United States. Some of these phages include SalmoFresh and EcoShield produced by Intralytix and PhageGuard S and PhageGuard E produced by Micreos (Carter et al., 2012; Moye et al., 2018; Pietracha & Misiewicz, 2016; Sukumaran et al., 2016; Tolen et al., 2015). Table 1.1 details some of the phage cocktails commercially available in the United States. These commercial products are widely marketed for meat, poultry, seafood, fruits and vegetables, and food processing

environments. Many commercial phage cocktails are GRAS, and because phages can be used as a food processing aid (Ahmadi et al., 2020), they are also considered clean label.

Table 1.1 Select Phage Cocktails for *Salmonella* and STEC Control Available Commercially in the United States

Product Name	Company	Target Organism	Number of Phages
EcoShield	Intralytix	STEC	3
PhageGuard E	Micreos	STEC	2
PhageGuard S	Micreos	*Salmonella*	2
SalmoFresh	Intralytix	*Salmonella*	6
SalmoPro	PhageLux	*Salmonella*	2
Secure Shield E1	FINK TEC	STEC	6

In addition to the commercially available cocktails, various research groups have assessed the efficacy of novel *Salmonella* phage cocktails on a variety of foods, including tomatoes, lettuce, apples, melons, raw and cooked poultry, fruit juices, eggs, ground meat, and cheese. An extensive review on food challenge models for varying *Salmonella* phage cocktails has been composed by Oh & Park (2017). Notably, results from each study were variable, with log reductions in *Salmonella* ranging from 0.4 to 7 after phage addition. Storage temperature and the type of food matrix appear to play a role in how effective the phages were in reducing *Salmonella* levels. Various other factors including pH, sodium levels, exposure to UV light, interactions with other antimicrobials, surface immobilization, and interaction with food matrix components have all been reported to impact phage stability and performance (Adams, 1959; Ahmadi et al., 2020; Bao et al., 2015; Chibeu et al., 2013; Fister et al., 2016; Gill et al., 2006; Greer, 2005; Guenther et al., 2009; Guenther & Loessner, 2011; Guglielmotti et al., 2012; Jurczak-Kurek et al., 2016; Kajiura et al., 2001; Sharma et al., 2017). The complexity of food matrices can make predicting how well the cocktails will perform in a broad range of applications difficult. However, the reductions seen in studies to date show that phages are promising for *Salmonella* control.

1.6 Conclusions

Salmonella and STEC remain significant public health issues in the U.S. as two of the most common agents involved in foodborne illnesses each year. Many methods of control are employed, but some of these are less favorable for consumers, indiscriminate killers of microbes, or not compatible with raw and RTE foods. Phages have emerged as an increasingly popular alternative to control *Salmonella* and STEC in foods because they are selective, safe, and natural. Criticisms of phages primarily revolve around the inevitability of resistance emerging, but this may be addressed by using a phage cocktail with several phages targeting different receptors. A select few phages and phage cocktails have been assessed for their ability to control *Salmonella* and STEC in the food industry, with success varying by food product and storage conditions. Most phages remain undiscovered, so continuing to identify phages and develop cocktails that are effective against *Salmonella* and STEC will diversify the options available to prevent outbreaks.

Chapter 2: Isolation of Bacteriophages from Local Wastewater

2.1 Introduction

2.1.1 Salmonella and STEC as foodborne pathogens

Salmonella enterica subsp. *enterica* is a Gram-negative, rod-shaped bacterium. *S. enterica* is categorized into over 2500 groups, or serotypes, based on their antigenic structures. The O antigen is lipopolysaccharide (LPS), H antigens consist of flagella, and capsular polysaccharide makes up the Vi antigen, though this antigen is found in a select few serotypes, most notably *Salmonella* Typhi (Alikhan et al., 2018; Giannella, 1996). Of these over 2500 serotypes, only a select number have been frequently associated with human disease. Additionally, severity of infection varies with serotype (Jones et al., 2008). Prevalence of *Salmonella* serotypes vary in different geographic regions and in different food and animal commodities (Boore et al., 2015; Ferrari et al., 2019; Jackson et al., 2013; Scallan et al., 2011; Worley et al., 2018).

Escherichia coli are Gram-negative, rod-shaped bacteria. Like *Salmonella*, *E. coli* is classified into serotypes based on its antigens, O, H, and K. The O antigen is LPS, the H antigen is flagella, and the K antigen consists of capsular polysaccharides. Most *E. coli* are harmless and compose a normal part of a healthy human gut microbiome, but pathogenic Shiga toxin-producing *E. coli* (STEC) have acquired genes for producing Shiga toxin. Seven serotypes cause most of the foodborne STEC infections in the United States: O157:H7, O26, O111, O103, O121, O45, and O145 (Minnesota Department of Health, 2019).

2.1.2 Impact of Salmonella and STEC in the food system

Every year *Salmonella* is implicated in approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the United States alone, with food being the primary vehicle (Centers for Disease Control and Prevention [CDC], 2021a). Symptoms of salmonellosis manifest anywhere from six hours to twelve days after infection (CDC, 2021a). Gastroenteritis accompanied by diarrhea, abdominal pain and cramping, nausea, vomiting and headaches. The infections are generally self-limiting, with most symptoms subsiding in ten days or less (Eng et al., 2015). However, in some cases salmonellosis can result in development of bacteriemia or other invasive infection, particularly in children under five years of age, the elderly, and the immunocompromised. In other cases,

salmonellosis results in long-term effects, such as reactive arthritis that can remain for month to years after infection (CDC, 2021a). The large number of *Salmonella* infections result in an economic burden of approximately $3.7 billion annually in the United States (Hoffman et al., 2015).

STEC is estimated to cause over 265,000 illnesses, 3,600 hospitalizations, and 30 deaths each year in the United States (Scallan et al., 2011). Symptoms can develop anywhere between one to ten days after exposure to STEC, but most commonly develop in three to four days (CDC, 2021b). Around 5-10% of those with a STEC infection will develop Hemolytic Uremic Syndrome (HUS), which may result in renal failure and death (CDC, 2021b). Among those most susceptible to STEC are children under the age of five, the immunocompromised, and the elderly. The annual economic burdens attributed to O157:H7 STEC and non-O157:H7 STEC are estimated at $271 million and $27 million respectively (Hoffman et al., 2015).

2.1.3 Food attributions and traditional control of Salmonella and STEC

Salmonella is unique from other foodborne pathogens because it is attributed with a larger range of commodities than others. Approximately 75% of *Salmonella* outbreaks are distributed between seven categories: chicken, seeded vegetables, pork, fruits, other produce, eggs, and turkey (Interagency Food Safety Analytics Collaboration, 2020). For STEC, row vegetables are the most common vehicle for foodborne outbreaks, followed by beef (Interagency Food Safety Analytics Collaboration, 2020; Pires et al., 2011). This is likely because cows serve a reservoir for STEC (Etcheverría & Padola, 2013), meaning that fields of row vegetables could be contaminated from nearby cattle facilities, and beef can be contaminated during slaughter.

Traditionally used methods of control for pathogens such as *Salmonella* and STEC in food and on food-contact surfaces include use of chemical antimicrobial agents (Beuchat & Ryu, 1997), high pressure processing (Argyri et al., 2018), cold atmospheric gas plasma technology (Fernández et al., 2013), irradiation (Brito et al., 2002), and other physical and thermal processes. However, concerns with these methods have been raised over antimicrobial resistance (Barrow & Soothill, 1997; Bower & Daeschel, 1999; Davidson & Harrison, 2002), potential environmental impacts of chemical antimicrobials

(Banach et al., 2015), alteration of organoleptic properties (Brito et al., 2002; Moye et al., 2018), and a lack of consumer acceptance of irradiated products (Wheeler et al., 1999). Moreover, many of these methods are incompatible with raw and ready-to-eat foods, including raw meat, poultry, and fresh fruits and vegetables. Bacteriophages have been proposed as an alternative method of control of pathogens in food and agriculture applications, especially for raw and ready-to-eat foods.

2.1.4 Bacteriophages as a biocontrol agent

Bacteriophages, or phages, are viruses that are specific to bacteria. Phages are massively abundant on Earth, with the number of phage virions estimated to exceed 10^{31} (Bergh et al., 1989; Brüssow & Hendrix, 2002; Hatfull, 2008b). Phages are ubiquitous: they can be found in any environment capable of supporting bacterial growth (Campbell, 1961). Phages have two primary lifecycles, the lytic cycle and the lysogenic cycle. Both cycles begin with the phage particle adsorbing onto a receptor on the host's surface, irreversibly binding, and injecting their DNA into the host cell. Phages undergoing the lytic cycle then hijack the host's replication machinery to produce more phage particles. The lytic cycle ends with lysis and death of the host cell and the release of progeny phages. Phages undergoing the lysogenic cycle will incorporate their DNA into the host's genome rather than reproducing and lysing the host, though they can enter the lytic cycle at a different time. Virulent phages are strictly lytic and cannot enter the lysogenic cycle, whereas temperate phages can enter either cycle, though they more commonly participate in the lysognic cycle. During the lytic cycle, phages may mistakenly incorporate host DNA into a capsid. Such phages are known as transducing phages. When these phages bind to another host, they will inject DNA from the previous host into the cell. The new host cell may integrate this DNA into its genome. This process is known as generalized transduction, which can be performed by either temperate or lytic phages. In lysogenic conversion, a phage with a genome containing a gene from a different host undergoes the lysogenic cycle, and the phage genome (containing a gene previously found in another host) is incorporated into the new host. Perhaps the most significant example of this for the food industry is the lysogenic conversion of non-pathogenic *E. coli* to Shiga-toxin producing *E. coli* by a temperate phage carrying genes encoding for Shiga-toxin production (Khalil et al., 2016; Monteiro et al., 2019; Shaikh & Tarr, 2003). Because of

concerns over lysogenic conversion by temperate phages resulting in pathogens that are less responsive to current measures of control, phages for use in the food and agriculture sectors should be strictly lytic phages (Harrison & Brockhurst, 2017; Oliveira et al., 2015; Pirnay et al., 2015).

2.1.5 Advantages of phages as biocontrol agents of foodborne pathogens

Interest in using phages for biocontrol of pathogens in food and agriculture has grown in recent years due to the beneficial properties of phages. Phages are generally specific for certain species of bacteria, often only infecting a certain subpopulation of that species, which allows for the preservation of desirable microbes in the food system while managing the pathogen population. Phages can be found in any environment that supports their host bacteria, meaning they are naturally found in foods (Sillankorva et al., 2012). Ingestion studies of phages have indicated that they are safe for human consumption (Alisky et al., 1998; Bruttin & Brüssow, 2005; McCallin et al., 2013b). In fact, there are commercially available phages that have received GRAS status in the United States (Cairns & Payne, 2008; Hagens & Loessner, 2010; Moye et al., 2018; O'Flaherty et al., 2009; Spricigo et al., 2013). Yet another benefit of phages for biocontrol is that phages are not known to affect the organoleptic properties of foods (Otto et al., 2011; Perera et al., 2015; Pietracha & Misiewicz, 2016). Moreover, they can be used in organic products (Moye et al., 2018), which are growing in popularity with consumers (Reganold & Wachter, 2016). One study has even indicated that consumers may be willing to pay a premium for phage-treated produce after learning about the utility and safety of phages (Naanwaab et al., 2014).

2.1.6 Criticisms of adopting phages for pathogen biocontrol

While the adoption of phages for biocontrol in the food and agriculture industries shows many promising benefits, the industries have mixed opinions about utilizing phages (de Melo et al., 2018). Frequently cited concerns include worries about phages persisting in the food processing environments and spreading between facilities (Fister et al., 2016; Ly-Chatain, 2014; Meaden & Koskella, 2013), phages interfering with testing for pathogens and producing false negatives (Brown-Jaque et al., 2016; Muniesa et al., 2005), and re-growth of pathogens after phage treatment (Endersen et al., 2013; McLean et al., 2013). However, the greatest source of hesitancy to adopt phages for biocontrol is

the concern over phage-resistant mutants arising (Andreatti Filho et al., 2007; Cairns & Payne, 2008; Carey-Smith et al., 2006; Greer, 2005; Hong et al., 2016). Despite these criticisms, phage cocktails have shown promise in reducing pathogen levels in food applications.

2.1.7 Phage cocktails as a solution

To prevent phage-resistant mutants and to increase the range of hosts that can be targeted, developing cocktails of multiple phages that recognize different host receptors or have different means of evading host defenses has been proposed (Andreatti Filho et al., 2007; Bai et al., 2019; Chan et al., 2013; Goodridge & Bisha, 2011; Hudson et al., 2005; Jason & Paul, 2009; Kim & Ryu, 2012; Kortright et al., 2019). There are some *Salmonella* and STEC phage cocktails available on the market, and various studies have discussed novel phage cocktails able to reduce *Salmonella* levels in food matrices (Moye et al., 2018; Oh & Park, 2017). However, there are a limited number of such phages that have been characterized and combined into cocktails for biocontrol. A greater diversity of phages is beneficial for use in cases where resistance has developed to the available phages. In this study, wastewater from local facilities was utilized to isolate novel bacteriophages. The host ranges of the phages were assessed by the phages' ability to lyse a variety of *Salmonella* serotypes of concern. A phage cocktail with potential for *Salmonella* control in food settings was developed from six phages with complimentary host ranges that encompass all serotypes tested. While *Salmonella* biocontrol was the primary objective in this study, the cocktail was also tested for its ability to lyse the seven most common STEC serotypes because the phages also lysed a non-pathogenic *E. coli* strain.

2.2 Materials and Methods

2.2.1 Bacterial strains, culture conditions, and wastewater preparation

Nineteen *Salmonella* strains, seven STEC strains, and one nonpathogenic *Escherichia coli* K-12 strain, from several sources (Table 2.1) were used in the enrichment, isolation, and testing of unique bacteriophages. Strains used for this study were provided by Paradigm Diagnosic Inc. (PDX), the *Salmonella* Genetic Stock Centre in Calgary, Alberta, Canada (SGSC), the Minnesota Department of Health (MDH), Jay Hinton at the University of Liverpool, UK, and Lidija Truncaite at Vilnius University, Lithuania. All

strains Bacteria strains were cultured at 37ºC for 16-18 hours on Luria-Bertani (LB) broth or LB agar plates.

Wastewater primary influent samples were collected from the Eagle's Point Wastewater Treatment Plant (Cottage Grove, MN, USA) and the Metropolitan Wastewater Treatment Plant (Saint Paul, MN, USA). The large particulate matter was removed from the wastewater samples by filtration through Whatman No. 1 filter paper. Following this, the samples were vacuum filtered using 0.22 um pore size filters (Millipore) to remove existing bacterial cells. The filtrates were stored at 4ºC until used.

Table 2.1 *Salmonella* and *E. coli* strains used for phage host range assessments

Serotype	Strain	Source
S. Agona	ATCC 51957	PDX
S. Bareilly	PDX BB3	PDX
S. Enteritidis	SGSC 2475	SGSC
S. Hadar	PDX CC12	PDX
S. Heidelberg	PDX AC2	PDX
S. Infantis	I2018008804-1	MDH
S. Javiana	PDX CD13	PDX
S. Kentucky	PDX AB7	PDX
S. Mississippi	PDX BD2	PDX
S. Montevideo	ATCC 8387	PDX
S. Muenchen	PDX BA12	PDX
S. Newport	ATCC 6962	PDX
S. Oranienburg	PDX AE15	PDX
S. Reading	E2018018984	MDH
S. Saint Paul	PDX CD7	PDX
S. Thompson	PDX CB3	PDX
S. Typhimurium	4/74	Jay Hinton
S. Typhimurium	LT2 SGSC 1412	SGSC
S. Typhimurium	LT2 SGSC 288 (*waaL* mutant)	SGSC
E. coli O103	CDC 06-3008	PDX
E. coli O111:H8	CDC 2010 C-3114	PDX
E. coli O121	PDX ED3	PDX
E. coli O145:NM	CDC 99-3311	PDX
E. coli O157:H7	EDL933 ATCC 43895	ATCC
E. coli O26:H11	CDC 03-3014	PDX

E. coli O45:H2	CDC 00-3039	PDX
E. coli K-12	MG1655	Lidija Truncaite

2.2.2 Bacteriophage enrichment and isolation

Five milliliters of wastewater filtrate were combined with 0.1mL of a *Salmonella* strain overnight culture and 45mL of LB broth in a 250mL conical flask. The mixture was incubated for 37ºC with shaking for 16-18 hours to enrich viable phages on this host strain. The phage enrichment was syringe filtered through a 0.22um pore size filter.

Individual phages were isolated using the double agar overlay plaque assay (Figure 1). A mixture of phages was cultivated by combining 0.1mL of phage enrichment lysates, 0.1mL of an overnight culture of a selected *Salmonella* strain for phage propagation, and 4mL of molten 0.35% w/v LB agar. This mixture was poured onto a 1.5% w/v LB agar plate and allowed to solidify. After incubation at 37ºC for 16-18 hours (Figure 2), individual plaques were picked and streaked onto 1.5% LB w/v agar plates. Four milliliters of molten 0.35% w/v LB agar containing 0.1mL of the propagation strain were overlayed onto the streaked plate. After solidification of the agar overlay, the plate was incubated for 37ºC for 16-18 hours. The streaking processes was repeated three to five times, until a uniform plaque morphology was achieved.

To create pure phage lysate solutions, a single isolated plaque was picked using a 1000uL pipette tip and aspirated into 10mL of LB broth containing 0.1mL of the propagation strain. After incubation at 37ºC with shaking for 16-18 hours, phage lysates were syringe filtered using 0.22um pore size filters. Phage lysates were stored at 4ºC.

2.2.3 Determination of phage phenotypic host ranges

Phage host ranges were determined phenotypically using spot on the lawn plaque assay (Figure 2.3). Four milliliters of molten 0.35% w/v LB agar was combined with 0.1mL of bacterial overnight culture and poured onto a 1.5% w/v LB agar plate. While the agar solidified, phage lysates were serially diluted in SM buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 8mM $MgSO_4$). Ten microliter aliquots of serially diluted phage lysate were spotted onto the agar overlay. After the spots dried, the plates were incubated at 37ºC for 16-18 hours. Phages were assessed on their ability to form zones of lysis and/or plaques (Figure 3C) on each individual strain, demonstrating the host range of each phage

(Refer to Table 1 of Appendix A). All isolated phages were tested for their ability to lyse 19 *Salmonella* strains representing 17 serotypes and one strain of *E. coli* K-12. Additionally, the phages selected for a cocktail were tested against 7 STEC strains representing 7 different serotypes.

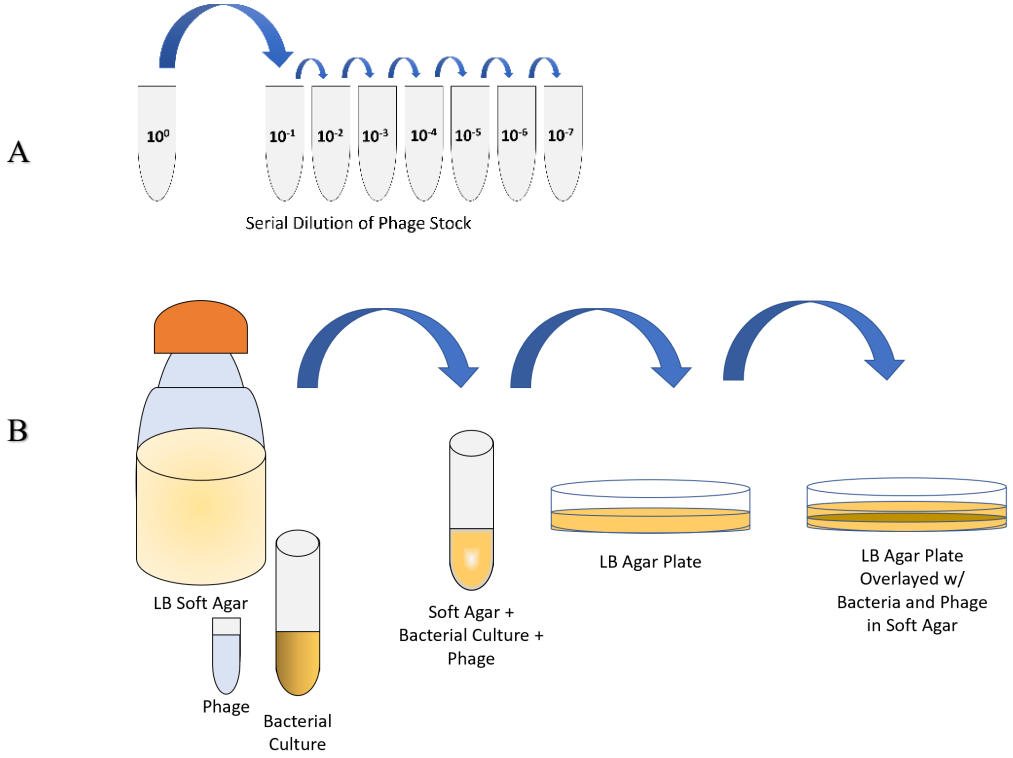


Figure 2.1 Double agar overlay plaque assay. (A) Phage solution is serially diluted in SM buffer. (B) Diluted phage, culture of host bacteria, and LB soft agar are combined and poured over an LB agar plate.

2.2.4 Selection of phages for cocktail

Phenotypic *Salmonella* host ranges of individual phages were compared based on the results of the spot on the lawn assays. Phages with distinct host ranges that lysed a broad variety of serotypes were selected. Six phages were selected to be combined into a cocktail that could act synergistically against all 17 tested *Salmonella* serotypes based on their complimentary host ranges.

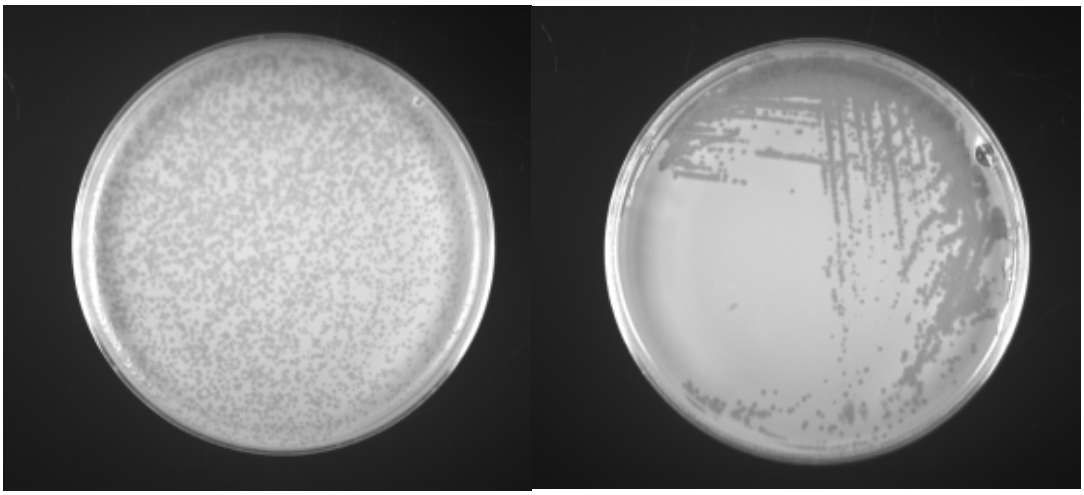
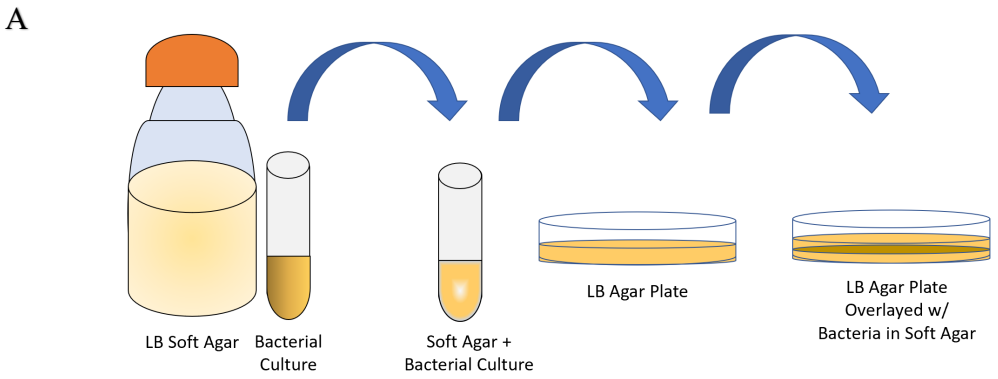
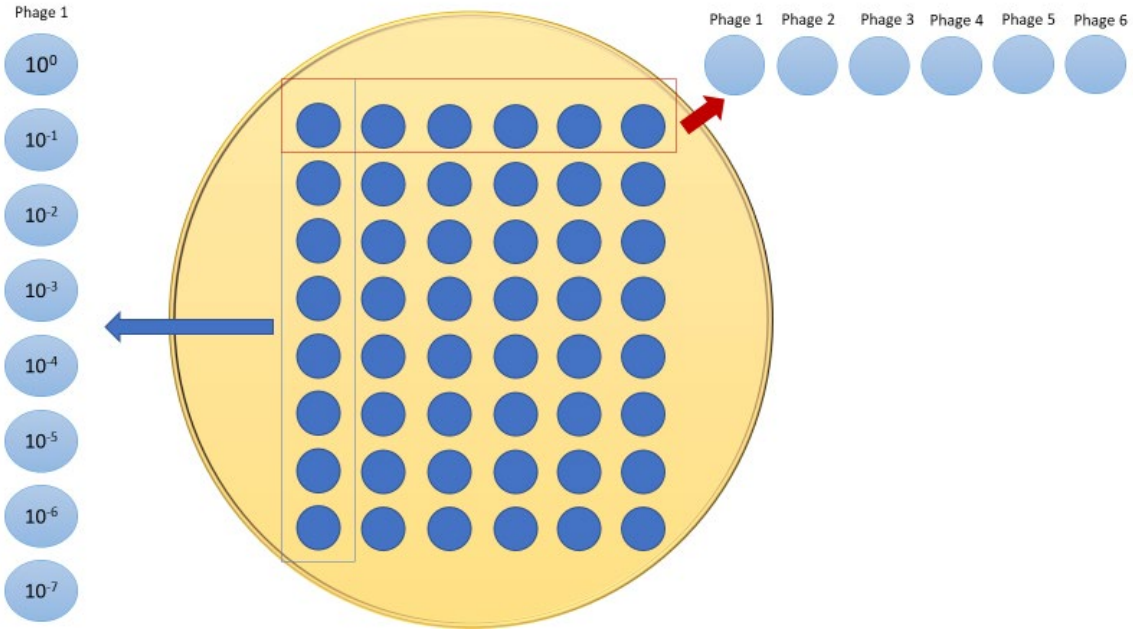


Figure 2.2 Example plaque assay plates. Left: Double agar overlay plaque assay for phage enrichment samples. A mixture of plaque morphologies is found, suggesting a variety of phages are present. Right: Single plaque picked and streaked out to isolate.



B


Phage 1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Phage 1
Phage 2
Phage 3
Phage 4
Phage 5
Phage 6

C

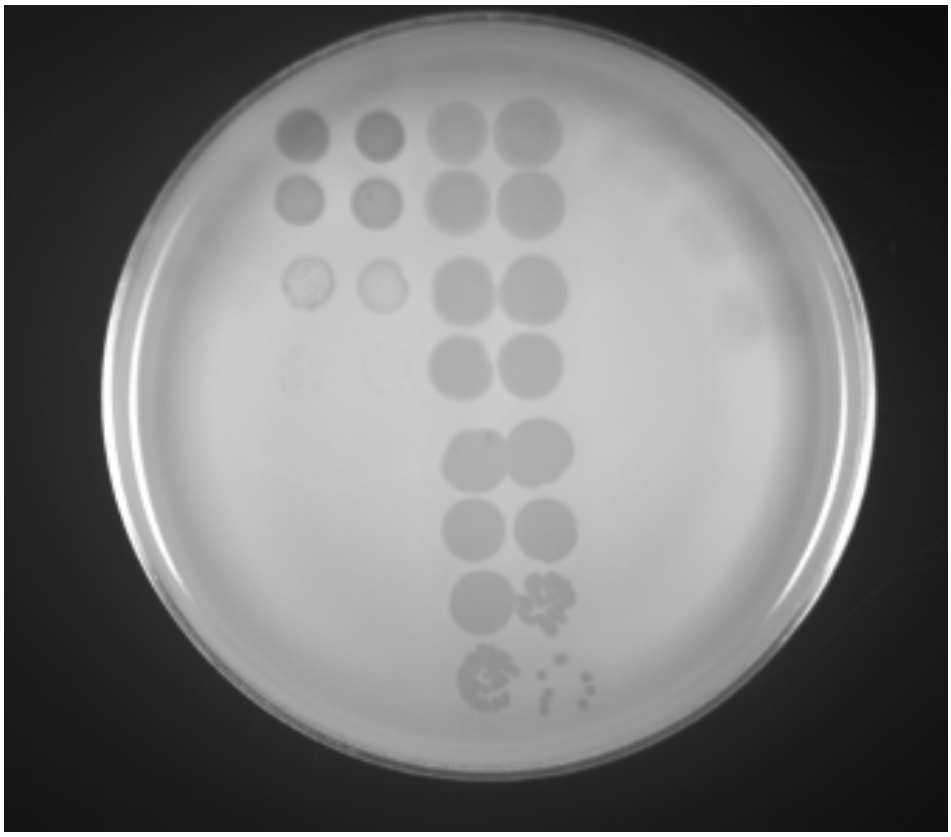


Figure 2.3. Spot on the lawn plaque assay. (A) Bacteria host culture and LB soft agar are combined and poured over an LB agar plate. (B) Phages are serially diluted and spotted onto cooled agar overlay. (C) Example spot on the lawn plaque assay. The leftmost columns show lysis zones but no individual plaques, thus this strain is deemed partially sensitive to these two phages. The center two columns show zones of lysis as well as individual plaques at lower titer, indicating that this strain is sensitive to the two phages. The phage on the far right is not observable, as no zones of lysis or plaques formed. The strain is considered resistant to this phage.

2.3 Results and Discussion

2.3.1 Bacteriophage isolation

While there are some commercial *Salmonella* phages available for use in the food industry, as summarized in a review by Oh and Park (2017), there is not much diversity in phages currently used in industry. The total number of phage virions present on Earth is estimated to be in the range of 10^{31} (Bergh et al., 1989; Brüssow & Hendrix, 2002; Hatfull, 2008b), many of which remain undiscovered (Cobián Güemes et al., 2016; Dutilh et al., 2014). Isolation and characterization of novel phages from the wealth of virions available increases the options to combat *Salmonella* in the food and agriculture industries. Wastewater from local Minnesota treatment facilities was selected as a source to hunt for these phages because novel phages have been successfully isolated from wastewater in other locations (Akhtar et al., 2014; Edham et al., 2017; Jurczak-Kurek et al., 2016; Weber-Dąbrowska et al., 2016). A total of 27 bacteriophages were successfully isolated from the wastewater samples. Phages were selected based on plaque morphologies that appeared unique when plating enrichment samples on select propagation strain(s) and the ability to withstand the isolation process. Some plaques did not yield stable phages in the conditions used to isolate the phages, so these phages were not assessed further. The 27 successfully isolated phages were enriched and propagated on a variety of *Salmonella* strains, as summarized in Table 2.2.

Table 2.2. Summary of isolated phages, strains used to enrich the phages, and the strains used for isolation and propagation, and wastewater source facilities

Phage	Enrichment Strains		Propagation Host Strains		Water Source
	Strain	*Source*	*Strain*	*Source*	
EH1	*S.* Typhimurium 4/74	Jay Hinton	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
EH2	*S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
			S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	
EH3	*S.* Enteritidis SGSC 2475	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
			S. Enteritidis SGSC 2475	SGSC	
			S. Reading E2018018984	MDH	
			S. Infantis I2018008804-1	MDH	
EH4	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	*S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	
EH5	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	*S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC	
EH6	*S.* Typhimurium 4/74	Jay Hinton	*S.* Typhimurium 4/74	Jay Hinton	Eagle's Point & Metropolitan Mixed
	S. Kentucky PDX AB7	PDX	*S.* Kentucky PDX AB7	PDX	
	S. Montevideo ATCC 8387	PDX	*S.* Montevideo ATCC 8387	PDX	

EH7	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
EH8	*S.* Typhimurium 4/74	Jay Hinton	*S.* Typhimurium 4/74	Jay Hinton	Eagle's Point
EH9	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium 4/74	Jay Hinton	Eagle's Point
EH10	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412	SGSC	Eagle's Point
EH11	*S.* Typhimurium LT2 SGSC 1412 *S.* Enteritidis SGSC 2475 *S.* Reading E2018018984 *S.* Infantis I2018008804-1	SGSC SGSC MDH MDH	*S.* Typhimurium LT2 SGSC 1412 *S.* Enteritidis SGSC 2475 *S.* Reading E2018018984 *S.* Infantis I2018008804-1	SGSC SGSC MDH MDH	Eagle's Point
EH12	*S.* Reading E2018018984	MDH	*S.* Typhimurium LT2 SGSC 1412 *S.* Enteritidis SGSC 2475 *S.* Reading E2018018984 *S.* Infantis I2018008804-1	SGSC SGSC MDH MDH	Eagle's Point
EH13	*S.* Reading E2018018984	MDH	*S.* Typhimurium LT2 SGSC 1412 *S.* Enteritidis SGSC 2475 *S.* Reading E2018018984 *S.* Infantis I2018008804-1	SGSC SGSC MDH MDH	Eagle's Point
EH14	*S.* Infantis I2018008804-1	MDH	*S.* Enteritidis SGSC 2475	SGSC	Eagle's Point

EH15	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH16	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH17	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH18	*S.* Typhimurium LT2 SGSC 1412	SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH19	*S.* Typhimurium LT2 SGSC 288	SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH20	*S.* Typhimurium LT2 SGSC 288	SGSC	*S.* Typhimurium LT2 SGSC 1412 *S.* Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC SGSC	Eagle's Point
EH21	*S.* Kentucky PDX AB7	PDX	*S.* Kentucky PDX AB7	PDX	Eagle's Point & Metropolitan Mixed

EH22	*S.* Kentucky PDX AB7	PDX	*S.* Kentucky PDX AB7	PDX	Eagle's Point & Metropolitan Mixed
EH23	*S.* Thompson PDX CB3	PDX	*S.* Thompson PDX CB3	PDX	Eagle's Point & Metropolitan Mixed
EH24	*S.* Thompson PDX CB3	PDX	*S.* Thompson PDX CB3	PDX	Eagle's Point & Metropolitan Mixed
EH25	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	Eagle's Point & Metropolitan Mixed
EH26	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	Eagle's Point & Metropolitan Mixed
EH27	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	*S.* Typhimurium 4/74 *S.* Kentucky PDX AB7 *S.* Montevideo ATCC 8387	Jay Hinton PDX PDX	Eagle's Point & Metropolitan Mixed

2.3.2 Host range analysis on Salmonella

Prevalence of different *Salmonella* serotypes associated with human disease is highly variable with regard to commodity and geography (Boore et al., 2015; Ferrari et al., 2019; Jackson et al., 2013; Scallan et al., 2011; Worley et al., 2018). Determining the phenotypic host range of *Salmonella* phages provides insight on their individual utility and potential synergy to act against several serotypes when combined into a phage

cocktail. Host ranges for the isolated phages were assessed by their ability to create zones of lysis and individual plaques in spot on the lawn assays (Figure 3B) on several *Salmonella* strains from serotypes of interest for their roles in human disease or prevalence in food commodities (Table 2.1). Strains characterized as sensitive to a phage had zones of lysis at high phage titers and individual plaques forming at lower titers. Partially sensitive strains were identified by zones of lysis at higher titers but no evidence of individual plaques at reduced titers. Strains resistant to a phage showed no evidence of lysis. For a full comparison of strain sensitivity levels to each phage, see Table A1 of the Appendix. The number of *Salmonella* strains that were either sensitive or partially sensitive was tabulated for each phage (Table 2.3). A greater number of susceptible strains from different serotypes indicates a broader phage host range. The isolated phages' individual abilities to lyse various strains ranged between 32% and 95%. Most phages displayed a unique range of susceptible serotypes, but a few had nearly identical host ranges. This may indicate that these phages are clonal, very closely related, or utilize similar mechanisms of action. The phages EH1, EH7, EH8, and EH9 show similar host ranges to one another. EH1 and EH10, EH3 and EH14, EH16 and EH17, and EH22 and EH24 are pairs of phages that are potentially clonal or closely related. Two trios of phages, EH2, EH19, EH20 and EH6, EH25, and EH26 may also be made of up similar or clonal phages based on the phenotypic host ranges (Table A1).

Table 2.3. Percentage of *Salmonella* strains sensitive* to isolated phages

Phage	*Salmonella* Sensitivity
EH1	8/19 (42%)
EH2	13/19 (68%)
EH3	12/19 (63%)
EH4	9/19 (47%)
EH5	14/19 (74%)
EH6	14/19 (74%)
EH7	13/19 (68%)
EH8	13/19 (68%)
EH9	13/19 (68%)
EH10	9/19 (47%)

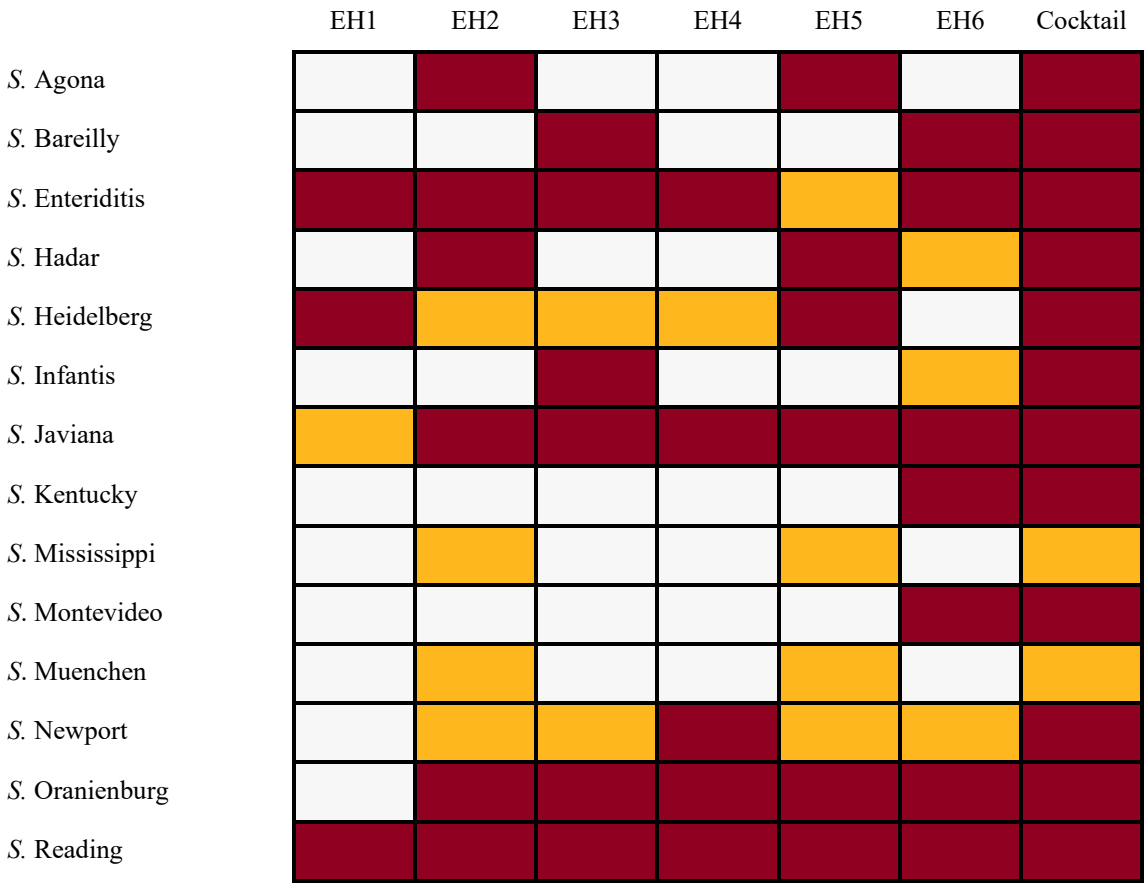
EH11	13/19 (68%)
EH12	8/19 (42%)
EH13	12/19 (63%)
EH14	9/19 (47%)
EH15	7/19 (37%)
EH16	11/19 (58%)
EH17	11/19 (58%)
EH18	7/19 (37%)
EH19	13/19 (68%)
EH20	13/19 (68%)
EH21	6/19 (32%)
EH22	6/19 (32%)
EH23	10/19 (53%)
EH24	9/19 (47%)
EH25	18/19 (95%)
EH26	12/19 (63%)
EH27	7/19 (37%)

*Sensitive defined as presence of zones of lysis and/or plaquing

2.3.3 Selection of phages for cocktail to combat Salmonella and STEC

Concerns over phage resistance are frequently cited as a concern for regular use of phages in biocontrol of foodborne pathogens (Andreatti Filho et al., 2007; Cairns & Payne, 2008; Carey-Smith et al., 2006; Greer, 2005; Hong et al., 2016). Genesis of phage-resistant mutants is considered inevitable due to selective evolutionary pressures in the host (Lenski, 1984). Developing cocktails of phages with unique host ranges is proposed to reduce the incidence of resistant mutants arising with phage treatment (Bai et al., 2019b; Chan et al., 2013; Goodridge & Bisha, 2011; Jason & Paul, 2009; Kortright et al., 2019). Because bacteria have developed numerous mechanisms for phage defense (de Jonge et al., 2019; Rostøl & Marraffini, 2019) and phages have, in turn, found means to overcome these defenses (Hampton et al., 2020), a combination of phages with unique host ranges and means of evading host defense systems could act synergistically to overcome defenses raised by resistant mutants. The host ranges of the isolated phages

were compared to identify a combination of phages that could lyse most serotypes used in this study. No phage individually had the capability to lyse all strains tested, but six phages were identified that had relatively broad host ranges that complimented each other to encompass all serotypes tested (Figure 2.4). Additionally, each of these phages lysed a non-pathogenic *E. coli* K-12 strain. Because of this, one strain of each of the seven serotypes of interest for Shiga-toxin producing *E. coli* (STEC) (Karmali, 2018) were tested for sensitivity to the cocktail phages (Table 2.1). Of the STEC serotypes tested, the phages could all lyse O157:H7, and a few phages could individually lyse O103, O145:NM, and O45:H2. A few *Salmonella* phages have been identified to capable of lysing STEC strains of the serotype O157:H7 (Bai et al., 2019b; Park et al., 2012), however phages in this study demonstrated the ability to lyse four unique STEC serotypes associated with human disease (Figure 2.4). In combination, the six phages identified may work synergistically to combat 17 different serotypes of *Salmonella* and 4 serotypes of STEC, or 100% and 57% of strains tested in this study (Table 2.4).



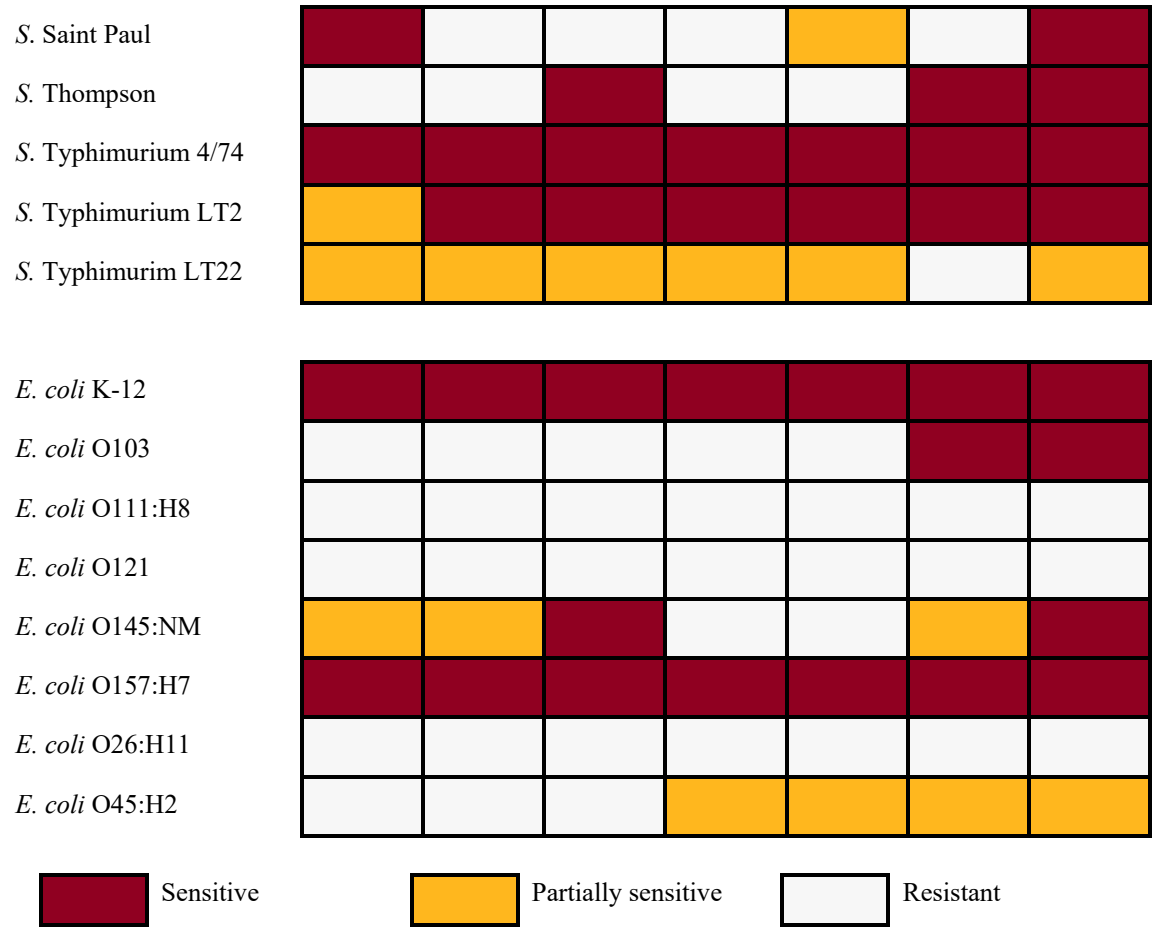


Figure 2.4. Phenotypic host ranges for 6 isolated phages alone and in combination. Sensitive strains were defined by zones of lysis and formation of individual plaques. Partially sensitive strains were defined by zones of lysis only. Strains were deemed resistant if evidence of lysis was absent.

Table 2.4. Table 1. *Salmonella* and STEC strains sensitive* to phages individually or in combination

Phage	*Salmonella*	STEC
EH1	8/19 (42%)	2/7 (29%)
EH2	13/19 (68%)	2/7 (29%)
EH3	12/19 (63%)	2/7 (29%)
EH4	9/19 (47%)	2/7 (29%)
EH5	14/19 (74%)	2/7 (29%)
EH6	13/19 (68%)	4/7 (57%)
Cocktail	**19/19 (100%)**	**4/7 (57%)**

*Sensitive defined as presence of zones of lysis and/or plaquing

2.4 Conclusion

Local Minnesota wastewater samples were utilized to successfully isolate 27 unique bacteriophages with potential for use to combat *Salmonella*. Six of these phages were selected for a putative cocktail for food and agriculture applications based on their complimentary host ranges. In combination, the six phages could lyse 19 *Salmonella* strains representing 17 serotypes. The selected phages were additionally able to lyse four strains of STEC representing 4 serotypes of concern, including O157:H7. Further characterization of these phages will be employed to explore their potential in controlling *Salmonella* in a food application. A cocktail of these six phages shows promise for reducing both major pathogens *Salmonella* and STEC in food and agriculture applications

Chapter 3: Novel Phage Cocktail Receptor Characterization

3.1 Introduction

3.1.1 Salmonella as a foodborne pathogen

Salmonella enterica subsp. enterica is a Gram-negative, rod-shaped bacterium. *S. enterica* is subdivided into over 2500 groups, known as serotypes, based on variations on the antigenic structures: lipopolysaccharide (LPS),flagella, and for some serotypes the capsular polysaccharide (Alikhan et al., 2018; Boyd et al., 1996; Brenner et al., 2000). Of these over 2500 serotypes, only a limited subset are frequently associated with human disease (Jones et al., 2008). *Salmonella* accounts for an estimated 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths annually in the United States, with most of these involving food as the vehicle for the pathogen (Centers for Disease Control and Prevention [CDC], 2021a). Salmonellosis symptoms can manifest anywhere from six hours to twelve days after infection (CDC, 2021a), but these infections are usually self-limiting with symptoms subsiding in ten days or less (Eng et al., 2015). Salmonellosis manifests as gastroenteritis, with symptoms commonly involving diarrhea, abdominal pain and cramping, nausea, vomiting, and headache. In some cases, more serious invasive *Salmonella* infections can occur, most commonly in children under the age of five, the elderly, and the immunocompromised. In rare cases, *Salmonella* infection can result in long-term effects such as reactive arthritis, which can last for months to years after the infection. (CDC, 2021a). Foodborne *Salmonella* is uniquely associated with a wide range of food sources. Around 75% of outbreaks are attributed to seven categories: chicken, seeded vegetables, pork, fruits, other produce, eggs, and turkey (Interagency Food Safety Analytics Collaboration, 2020). Moreover, *Salmonella* prevalence in the food system widely varies with regard to geographic location and commodity (Boore et al., 2015; Ferrari et al., 2019; Jackson et al., 2013; Scallan et al., 2011; Worley et al., 2018).

3.1.2 Traditional methods of Salmonella control in food systems

Common measures employed to control *Salmonella* in food systems involve chemical antimicrobials (Beuchat & Ryu, 1997), physical, and thermal processing methods. Frequently, high pressure processing, (Argyri et al., 2018), irradiation (Brito et al., 2002), and cold atmospheric plasma (Fernández et al., 2013) are utilized for control. However, concerns over these methods have been raised, including environmental impacts of

chemical antimicrobials (Banach et al., 2015), developing resistance to antimicrobials (Barrow & Soothill, 1997; Bower & Daeschel, 1999; Davidson & Harrison, 2002; Gebreyes & Thakur, 2005; Solano et al., 2002), and lack of consumer acceptance of irradiated products (Wheeler et al., 1999). Especially important is the concern over these methods impacting the organoleptic properties of the food (Brito et al., 2002; Moye et al., 2018). *Salmonella* is attributed with many raw and ready-to-eat foods, such as meat, poultry, and fresh fruits and vegetables, which are not compatible with many of these methods. In recent years, interest in using bacteriophages as a control measure for *Salmonella* in food applications(Arlet et al., 2006; P. García et al., 2008; Greer, 2005; Kazi & Annapure, 2016).

3.1.3 Bacteriophages for Salmonella biocontrol

Bacteriophages (phages) are viruses that are specific for bacteria. Phages can undergo different lifecycles, described below, but strictly virulent phages that lyse and kill the host cell are best for use in biocontrol of pathogens in the food industry (Harrison & Brockhurst, 2017; Oliveira et al., 2015; Pirnay et al., 2015). Incorporating phages for biocontrol of pathogens in foods and food-contact surfaces is advantageous because phages are highly specific (Atterbury et al., 2003b; Dalmasso et al., 2014), ubiquitous (Campbell, 1961; Hudson et al., 2005), naturally found in foods and are unlikely to alter food microflora (Moye et al., 2018; O'Sullivan et al., 2019), and do not alter the organoleptic properties of the foods (Otto et al., 2011; Perera et al., 2015; Pietracha & Misiewicz, 2016). Concerns over phages in the food industry are primarily limited efficacy, potential for causing false positive results in pathogen testing (Brown-Jaque et al., 2016; Muniesa et al., 2005), and, emergence of phage-resistant mutants (Andreatti Filho et al., 2007; Cairns & Payne, 2008; Carey-Smith et al., 2006; Geer, 2005; J. Hong et al., 2008). Phage cocktails containing a mixture of phages with different host ranges and recognizing a variety of host receptors is the proposed method of mitigating many of the present concerns (Andreatti Filho et al., 2007; Bai et al., 2019b; Chan et al., 2013; Goodridge & Bisha, 2011; Hudson et al., 2005; Jason & Paul, 2009; M. Kim & Ryu, 2012; Kortright et al., 2019).

3.1.4 Phage lifecycle and host receptors

Phages infect their hosts by first adsorbing to a specific receptor on the cell surface. Nearly any surface structure can serve as a receptor for phages. In Gram-negative bacteria, some presently identified receptors include LPS, other surface polysaccharides, flagella, and proteins such as BtuB, OmpC, OmpA, and TonB (Bertozzi Silva et al., 2016). Following this, irreversible binding occurs, and the phage injects its genetic material into the host cell. From this point, phages may either go through the lytic or lysogenic cycles. In the lytic cycle, the phage will seize control of the host's replication system and utilize it to produce new virions. The cycle culminates in the lysis of the host cell to release progeny phage. In contrast phages undergoing the lysogenic cycle incorporate their DNA into the host genome rather than reproducing and lysing the cell. This integrated phage, known as a prophage, may enter the lytic cycle at a later time. Virulent phages are strictly lytic, i.e., they can only go through the lytic cycle. Temperate phages may enter either the lysogenic or lytic cycle, though they more frequently enter the lysogenic cycle. Temperate phages may participate in specialized transduction, a process in which host genes nearby the phage genome are excised with the phage genome as it separates from the host (Griffiths et al., 2000; Monteiro et al., 2019). Specialized transduction can transfer virulence factors or antimicrobial resistance factors into a host's genome that were not found previously (Figueroa-Bossi et al., 2001; Monteiro et al., 2019). Because of this, phages for use in the food and agriculture sectors should be strictly lytic phages (Harrison & Brockhurst, 2017; Oliveira et al., 2015; Pirnay et al., 2015). When selecting lytic phages for a cocktail to control *Salmonella*, it is beneficial to select phages that recognize different receptors on the *Salmonella* surface. Changes in surface receptors are the most common cause of phage resistance (Mahichi et al., 2009), so having phages that recognize other receptors on the host allows for continued efficacy of the phage mixture. Commonly recognized *Salmonella* phage receptors include the LPS, flagella, and porins (Bai et al., 2019; Lindberg & Holme, 1969; Marti et al., 2013); however, many *Salmonella* phages have not yet had their receptors characterized (Bai et al., 2019; Gao et al., 2020; Islam et al., 2019; Petsong et al., 2019; Zschach et al., 2015), Identifying receptors for novel phages is important for developing an effective phage cocktail. In this study, putative receptors for six phages proposed for a novel cocktail for

Salmonella control were identified. Additionally, transmission electron micrograph images were generated for taxonomic classification of the phages.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions and primers

Eight *Salmonella* strains were utilized for bacteriophage propagation (Table 3.1). Strains used for this study were provided by Paradigm Diagnosic Inc. (PDX), the *Salmonella* Genetic Stock Centre in Calgary, Alberta, Canada (SGSC), the Minnesota Department of Health (MDH), and Jay Hinton at the University of Liverpool, UK.The strains were cultured at 37ºC for 16-18 hours on Luria-Bertani (LB) broth (Company information) or LB agar plates. For isolating phage-resistant mutants, a gene-knockout mutant library of *Salmonella* Typhimurium 4/74, created using the Lucigen EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit, was stored in LB broth containing 25% v/v glycerol in aliquots representing seven sub libraries. An aliquot of each of the seven sub libraries was thawed on ice directly prior to use. Primers used in this study are listed in Table 2.

Table 3.1. Cocktail phages used in this study and their host strain(s)

Phage	**Host(s)**	
	Strain	*Source*
EH1	*S.* Typhimurium LT2 SGSC 1412	SGSC
EH2	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC
EH3	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Enteritidis SGSC 2475	SGSC
	S. Reading E2018018984	MDH
	S. Infantis I2018008804-1	MDH
EH4	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC

EH5	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC
EH6	*S.* Typhimurium 4/74	Jay Hinton
	S. Kentucky PDX AB7	PDX
	S. Montevideo ATCC 8387	PDX

3.2.2 Phage sample preparations

Phages were propagated using the double agar overlay assay. Briefly, 0.1mL of phage lysate and 0.1mL of a culture of propagation strain(s) were mixed in with 4mL of molten 0.35% w/v LB agar. This mixture was poured onto a 1.5% w/v LB agar plate and allowed to solidify. After incubation at 37ºC for 16-18 hours, the top agar layer was mixed with 6mL of SM buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 8mM $MgSO_4$) in a conical tube and centrifuged at 5000rpm for 15 minutes. The supernatant was filter-sterilized using a 0.22um pore size syringe filter (Millipore). Filtered phage samples were stored at 4ºC.

3.2.3 Phage-resistant mutant isolation

Phages were plated on the gene-knockout mutant library to identify mutants resistant to the phages due to loss of the phage receptor as a result of EZ-Tn5™ <KAN-2> insertions. Thawed mutant sub library aliquots and a culture wild-type of *Salmonella* Typhimurium 4/74 were diluted in LB broth to an approximate concentration of 10^7 CFU/mL each. Each phage lysate was plated on the individual sub libraries and wild-type 4/74 using the double agar overlay assay. For each phage, five colonies from the sub library with the highest resistance rate compared to wild type were picked and streaked three times for isolation. Colonies were then cultured in LB broth overnight and were tested for true resistance to the phage using a spot on the lawn plaque assay: 0.1 mL of the cultured mutant was combined with 4mL of 0.35% LB agar. The mixture was poured over an LB agar plate. The associated phage was serially diluted in SM buffer, and 10uL spots of each dilution were pipetted onto the solidified agar plate. After 16-18 hours of incubation at 37ºC, the mutants were checked for signs of lysis. If no lysis was found, the mutant was determined to be resistant to the phage.

3.2.4 Phage receptor identification

Two mutants for each phage were selected to undergo random-primed PCR, as described by Fineran et al. (2005), to amplify the gene in which EZ-Tn5™ <KAN-2> was inserted to form the mutant. Briefly, colony PCR was performed using three primers with random sequences (APP_A1, APP_A2, APP_A3) expected to bind to various positions and a transposon specific primer that faces outward from Kan2 (Kan2_5A or Kan2_3A). A second PCR reaction was carried out on the products from the first reaction using a primer that anneals to the 5' end of the product of the random primers (APP_B) and a nested transposon specific primer that anneals to the 3' end of the product (Kan2_5B or Kan2_3B). Thermocycler conditions for all PCR reactions were as follows:

1. 94ºC for 3 minutes
2. 94ºC for 15 seconds
3. 42ºC for 30 seconds
4. 72ºC for 3 minutes

Repeat steps 2, 3 and 4 five times, increasing the temperature of step 3 by 1ºC per cycle

5. 94ºC for 15 seconds
6. 60ºC for 30 seconds
7. 72ºC for 3 minutes

Repeat steps 5, 6 and 7 24 times

8. 72ºC for 7 minutes

The second PCR products were sent for Sanger sequencing using the nested transposon primer (ACGT Inc., Wheeling, IL). Primers used for random-primed PCR are described in Table 3.2. Transposon insertions sites were identified using NCBI BLASTN against *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 4/74 (NCBI taxid:909946; nucleotide accession number NC_016857.1) in the nucleotide collection database.

Table 3.2. Primers used for this study

Primer	Function	Annealing Region	Sequence (5' to 3')
APP_A1	Random Primer	Unknown	GACCACACGTCGACTAGTGCNNNNNNNNNNTCTAC
APP_A2	Random Primer	Unknown	GACCACACGTCGACTAGTGCNNNNNNNNNNACGCC
APP_A3	Random Primer	Unknown	GACCACACGTCGACTAGTGCNNNNNNNNNNGATAC
APP_B	Random Primer Compliment	5' End of Random Primers	GACCACACGTCGACTAGTGC

KAN2_5A	Transposon-Specific Primer	5' End of Kan2 Facing Out	GAATATGGCTCATAACACCCCTTGTATTACTG
KAN2_5B	Transposon-Specific Nested Primer	3' End of KAN2_5A	CTTGTGCAATGTAACATCAGAGATTTTGAG
KAN2_3A	Transposon-Specific Primer	3' End of Kan2 Facing Out	CCAACTGGTCCACCTACAACAAAG
KAN2_3B	Transposon-Specific Nested Primer	3' End of Kan2_3A	CAAAGCTCTCATCAACCGTGGC

3.2.5 Phage TEM imaging and classification

Transmission electron microscopy was used to view the morphology of the phage virions. Filtered lysates of each phage at a titer of approximately 10^9 PFU/mL each were submitted to the University of Minnesota Imaging Center (Saint Paul, MN). Gail Celio prepared and imaged the phage samples as follows. 10uL aliquots of phage lysate were placed onto a 200-mesh formvar/carbon-coated copper grid. Remaining samples were wicked away from the grid after 30 seconds using a piece of filter paper. Phages were stained on the grid using 10uL of 0.5% phosphotungstic acid (pH 7). After two minutes the stain was wicked away using a piece of filter paper. Following this, the grid was air-dried for 5 minutes. Phages were imaged at 60,000x magnification using a JEOL JEM-1400Plus transmission electron microscope at 60kV. An Advanced Microscopy Techniques XR16 camera was used to record the images of the phages using AMT Capture Engine software ver. 7.0.0.187. Phages were classified using the updated International Committee on Taxonomy of Viruses (ICTV) guidelines, summarized in a review by Dion et al. (2020).

3.3 Results and Discussion

3.3.1 Phage receptor identification

Identifying receptors of phages intended for use in biocontrol of pathogens helps to identify which phages may work best together due to having different, widely conserved receptors can reduce the incidence of resistance in food settings. Receptor identification gives insights into serotypes that are likely to be susceptible to the phage based on their surface structures, which may allow for specific targeting of serotypes of interest with phages. Incorporating phages known to have different, broadly conserved receptors

provides additional means of infection in the case of a mutation developing that restricts a phage from binding to its receptor. Despite the benefits of identifying phage receptors, many have not been characterized (Bai et al., 2019). Putative receptors for phages in a novel 6-phage cocktail for use against *Salmonella* were identified using gene-knockout mutants that were resistant to the phages due to EZ-Tn5™ <KAN-2> insertions in genes encoding for the receptor. After PCR amplification and Sanger sequencing, the genes with an EZ-Tn5™ <KAN-2> insertion. Insertions for phages EH1, EH2, EH3, and EH6 occurred at nucleotide 4370298, which is inside gene *btuB*. EH4 and EH5 had insertions at 4368990 and 4370599 respectively, which are also located in *btuB* but indicate independent *btuB* insertions (Table 3.3). *btuB* is a gene that encodes for a porin that aids in the diffusion of vitamin B12 into the host cell (Figure3.1). The BtuB protein is widely conserved, with *Escherichia coli* and *Salmonella btuB* genes showing high similarity (Wei et al., 1992). This may account for the ability of all six phages to lyse various *E. coli* serotypes (see Chapter 2). BtuB has been identified previously as a receptor for *Salmonella* phages (J. Hong et al., 2008; M. Kim & Ryu, 2011; Shin et al., 2012).

While BtuB is a recognized *Salmonella* phage receptor, this leaves room for uncertainty over why each of the six phages appears to have a unique host range despite sharing a common receptor. In some cases, phages with multiple receptors have been identified, such as phage SP6 which has two types of receptor binding proteins, which allow it to have a greater host range than a phage with only one identified receptor (Tu et al., 2017). T4 and some other phages require more than one receptor for infection. In the case of T4, the porin OmpC and LPS are coreceptors for infection. The phage can successfully infect its host when OmpC is present, but when OmpC is unavailable for the phage it may only infect a host with LPS terminating on a glucose residue (Kortright et al., 2020; Washizaki et al., 2016). Additionally, receptor identification is not the only factor that determines a phage host range. Other surface structures such as LPS on the cell can influence the ability of a phage to bind to a receptor (Heller & Braun, 1982; J. Wang et al., 2005). Different *Salmonella* serotypes and strains can vary in their defense mechanisms against the phage, some of which are activated after phages have bound to the receptor (de Jonge et al., 2019). Some phages have mechanisms of avoiding these host defenses (Samson et al., 2013b), making them effective while others with the same receptor may be rendered

useless. Even differences in transcriptional and translational processes in the host can determine the ability of a phage to successfully replicate and lyse the cell (Howard-Varona et al., 2018). Further investigation into other host range determinants will be useful into providing greater insight into the variation in host ranges between these phages.

Table 3.3. BLASTN Identified EZ-Tn5™ <KAN-2> Insertion Sites in *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 4/74 Genome and Putative Phage Receptors. Genome NCBI taxid:909946 and nucleotide accession number NC_016857.1.

Phage	Nucleotide Insertion Point	Gene	Gene Position in Genome	Gene Accession Number	Putative Receptor
EH1	4370298				
EH2	4370298				
EH3	4370298	*btuB*	4369549 to 4371393	CP002487	BtuB
EH4	4368990				
EH5	4370599				
EH6	4370298				

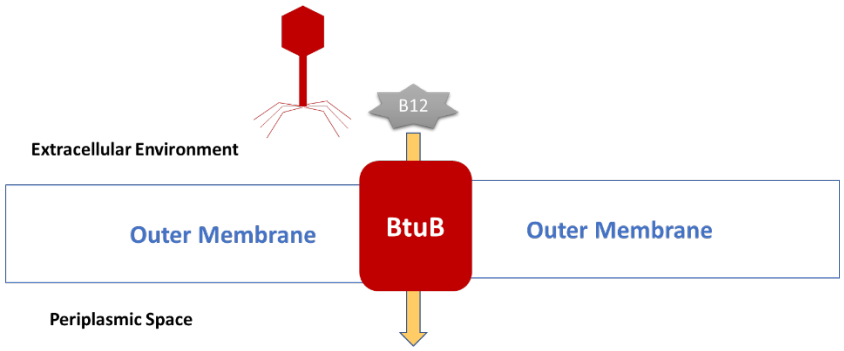


Figure 3.1. Putative phage receptor for cocktail phages. Putative receptor identified as BtuB for all phages. BtuB is a porin involved in importing vitamin B12 into the cell.

3.3.2 TEM images and taxonomic classification

Images returned of the six phages (Figure 3.2) showed that all six are tailed phages with polyhedral capsids. Phages EH1, EH2, EH3, and EH4 appear to have a long, flexible tail.

No contractile sheath is visible in the images of this phage. Phage EH5 displays a long, rigid tail which appears to be contractile. Phage EH6 also has a relatively long tail with a contractile sheath. In contrast to EH5, EH6 has small "star-like" appendages extending from the base of the tail. The other five phages are likely to have thin tail fibers near the base of the tail, but these are not readily visible in the TEM images. The 2018 ICTV guidelines group tailed phages into the *Caudovirales* order. Phages with a long, flexible tail that is not contractile belong to the *Siphoviridae* family. The *Myoviridae* family consists of phages with a long, inflexible tail that is contractile. The newly recognized family of *Ackermannviridae* have a morphology similar to Myoviridae, but they have a unique binding structure that consists of short structures with bulbous tips at the base of the tail (Dion et al., 2020). Based on these classification guidelines, EH1, EH2, EH3, and EH4 can be presumed to belong to the family *Siphoviridae*, EH5 belongs to the *Myoviridae* family, and EH6 likely belongs to the *Ackermannviridae* family, as summarized in Table 3.4.

3.4 Conclusion

A greater understanding of the mechanism of action for a bacteriophage is important for successfully designing a phage cocktail for biocontrol of *Salmonella*. An optimal phage cocktail contains several phages with unique, broad host ranges and multiple receptors to reduce the incidence of resistant *Salmonella*. In this study, six phages from a novel cocktail were TEM imaged to determine their taxonomy, and putative receptors were determined for the phages. All six phages were found to belong to the order *Caudovirales,* with four of the family *Siphoviridae*, one in the family of *Myoviridae*, and one belonging to *Ackermannviridae*. BtuB, a gene encoding for a B12 importing porin, was determined to be a putative receptor for all six phages. This leaves uncertainty about the reason for each phage having a unique host range. Further analysis to explore additional receptors or means of evading the hosts' intracellular defense mechanisms can provide more insight into the phages' efficacy on various serotypes and potential to suppress resistance

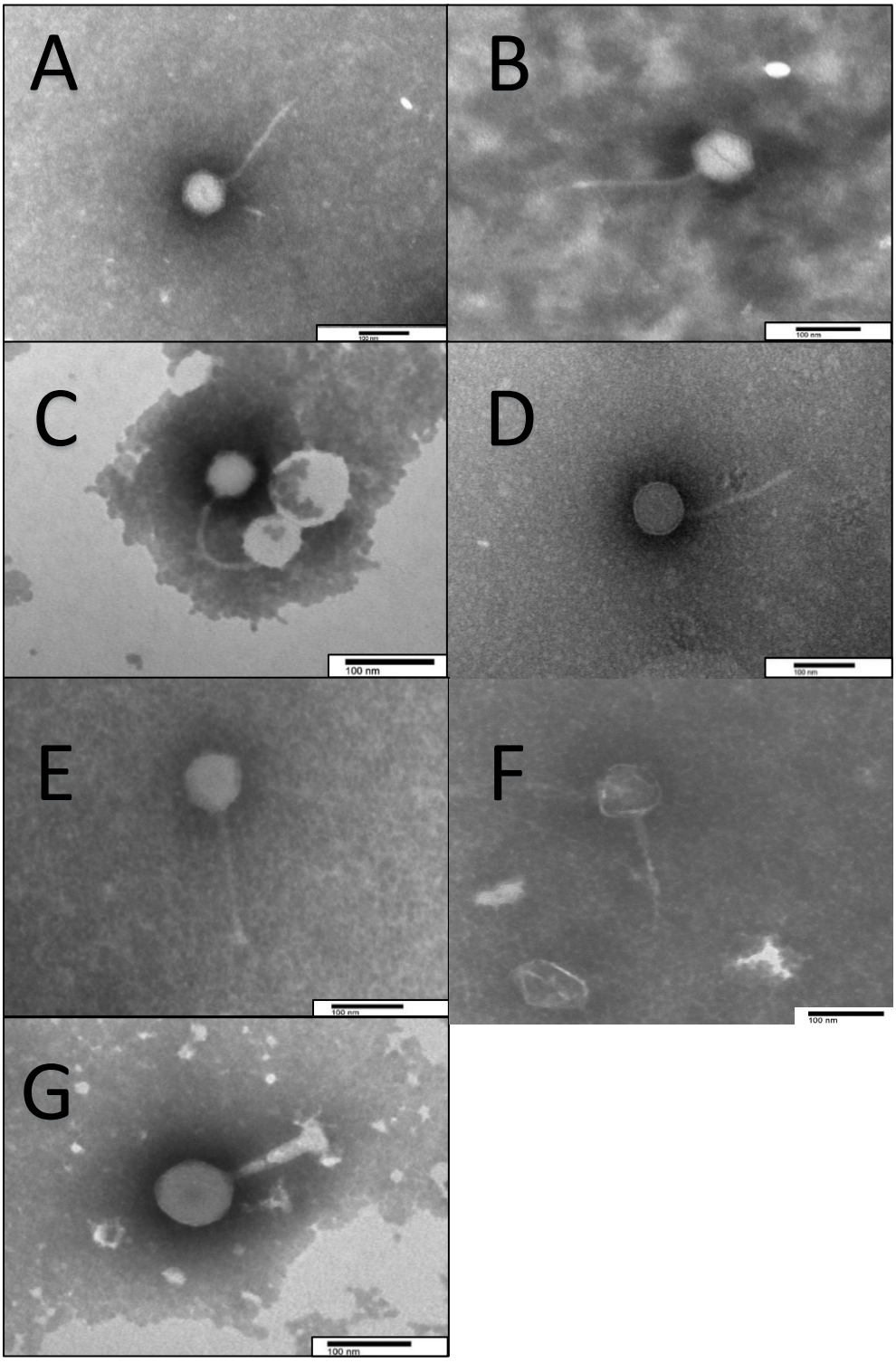


Figure 3.2. Transmission electron micrograph images of cocktail phages. Scale bar denotes 100nm in each image. (A) EH1 – Siphoviridae family. (B) EH2 – Siphoviridae

family. (C) EH3 – Siphoviridae family. (D) EH4 – Siphoviridae family. (E) EH5 – Myoviridae family. (F) EH5 with contracted tail (G) EH6 – Ackermannviridae family. Images courtesy of Gail Celio, University of Minnesota Imaging Centers.

Table 3.4. Taxonomic classification of phages

Phage	Order	Family	Defining Characteristics
EH1	*Caudovirales*	*Siphoviridae*	Long, flexible, non-contractile tail
EH2	*Caudovirales*	*Siphoviridae*	Long, flexible, non-contractile tail
EH3	*Caudovirales*	*Siphoviridae*	Long, flexible, non-contractile tail
EH4	*Caudovirales*	*Siphoviridae*	Long, flexible, non-contractile tail
EH5	*Caudovirales*	*Myoviridae*	Long, sheathed, contractile tail
EH6	*Caudovirales*	*Ackermannviridae*	Long, sheathed, contractile tail; short, filamentous adsorption structure at baseplate

Chapter 4: Reduction of *Salmonella* by Phage Cocktail in a Raw Chicken Breast Model

4.1 Introduction

4.1.1 Salmonella the foodborne pathogen

Salmonella enterica subsp. enterica is a Gram-negative, rod-shaped bacterium which is known as the causative agent of salmonellosis. In the United States alone, approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths are attributed to *Salmonella* every year. The majority of these cases involve food as the vehicle for transmission (Centers for Disease Control and Prevention [CDC], 2021a). Salmonellosis is characterized by gastroenteritis, accompanied symptoms often include diarrhea, nausea, vomiting, abdominal pain and cramping, and headache. These symptoms may begin anywhere from six hours to twelve days after infection (CDC, 2021a), and in most cases the symptoms will subside in ten days or fewer (Eng et al., 2015). In some severe cases, invasive *Salmonella* infections may occur; those under the age of five, the elderly, and the immunocompromised are most at risk. In other cases, long term effects such as reactive arthritis can remain for months to years after infection (CDC, 2021a). Preliminary reporting by Foodborne Diseases Active Surveillance Network for the year 2019 indicated that rates of *Salmonella* infections attributed to food sources did not decrease overall compared to the previous three years (Tack et al., 2020).

4.1.2 Salmonella in poultry

A wide range of foods are attributed to *Salmonella* outbreaks. Nearly 75% of *Salmonella* outbreaks are associated with seven food categories: chicken, seeded vegetables, pork, fruits, other produce, eggs, and turkey (Interagency Food Safety Analytics Collaboration, 2020), and each food is frequently associated with a particular serotype of *Salmonella*. *S. enterica* is subdivided into over 2500 groups known as serotypes. Serotypes are based on variations in the antigenic structures on the cell surfaces, which are lipopolysaccharide (LPS) and flagella, and, in a couple serotypes, capsular polysaccharide. Prevalence of *Salmonella* serotypes vary with geographic region and commodity (Boore et al., 2015; Ferrari et al., 2019; Jackson et al., 2013; Scallan et al., 2011; Worley et al., 2018). In poultry, *Salmonella* serotype Enteritidis is most common in Asia, Latin America, Europe, and Africa. In North America and Oceania, serotypes Kentucky, Typhimurium, and Sofia

are the most prevalent serotypes (Ferrari et al., 2019). While Kentucky is not yet known to have caused any outbreaks, it is important to closely monitor this serotype because of its prevalence (Ferrari et al., 2019).

Since 2015, at least nine outbreaks of *Salmonella* attributed to poultry have been reported. In 2021 so far, two outbreaks of *Salmonella* attributed to poultry have occurred, one from ground turkey and the other from raw frozen breaded stuffed chicken products (CDC, 2021a). Chickens are highly susceptible to gut colonization from *Salmonella* due to a wide variety of factors, including stress and rearing conditions, feed additives containing antimicrobials, transmission at hatching from infected parent, among others (Foley et al., 2008). With chicken consumption increasing in the U.S. from 22.4 pounds to 52.3 pounds per capita between 1970 and 2017 (Kantor and Blazejczyk, 2021), controlling *Salmonella* in chicken is as important as ever.

4.1.3 Bacteriophages for biocontrol of Salmonella

Bacteriophages are viruses that infect bacteria. Phage are extremely abundant on Earth with approximately 10^{31} virions (Bergh et al., 1989; Brüssow & Hendrix, 2002; Cobián Güemes et al., 2016). Bacteriophages act by adsorbing to the host cell and injecting their DNA into the host. Virulent phages will take control of the host cell's replication machinery to replicate. At the end of this lytic cycle, the host cell will be lysed by the phage, and the progeny phages will be released. Lytic phages have been proposed for biocontrol of pathogens in the food system because they are highly host-specific, ubiquitous (Campbell, 1961), safe for consumption (Alisky et al., 1998; Bruttin & Brüssow, 2005; McCallin et al., 2013b), low cost (Loc-Carrillo & Abedon, 2011; Nilsson, 2014), found naturally in foods (Atterbury et al., 2003b; Bao et al., 2015; Brüssow & Hendrix, 2002; Gautier et al., 1995; Sillankorva et al., 2012), and do not affect the organoleptic properties of foods (Otto et al., 2011; Perera et al., 2015; Pietracha & Misiewicz, 2016). Concerns over the use of phages in the food industry include potential for phages to remain in the processing environment (Fister et al., 2016; Ly-Chatain, 2014; Meaden & Koskella, 2013), producing false negatives in pathogen testing (Brown-Jaque et al., 2016; Muniesa et al., 2005), and, most importantly, genesis of phage-resistant mutants (Andreatti Filho et al., 2007; Cairns & Payne, 2008; Carey-Smith et al., 2006; Greer, 2005; Hong et al., 2008). However, resistance concerns may be

alleviated by developing phage cocktails containing multiple phages with different mechanisms to overcome host defenses (Andreatti Filho et al., 2007; Bai et al., 2019b; Chan et al., 2013; Goodridge & Bisha, 2011; Hudson et al., 2005; Jason & Paul, 2009; M. Kim & Ryu, 2012; Kortright et al., 2019).

A few phage cocktails are available for targeting *Salmonella* in the food industry (Pietracha & Misiewicz, 2016; Sukumaran et al., 2016; Tolen et al., 2015). Additionally, some research groups have released studies on phage cocktails intended to target *Salmonella,* many of which proved effective in reducing levels in raw chicken products (Moye et al., 2018; Oh & Park, 2017). Phage cocktails show promise for use in controlling *Salmonella* in raw chicken products.

4.1.4 Phage interactions with meat matrices and additives

Because of the complex and diverse nature of food matrices, phage efficacy varies with the type of food they are applied to. Parameters that affect phage efficacy in food matrices include pH, sodium levels, temperature, physical topology, and others (Greer, 2005; Guenther et al., 2009; Shannon et al., 2020). Concerns have been cited over varying phage efficacy in meat matrices. For example, a study involving a phage targeting *Listeria monocytogenes* found that reductions of the pathogen varied between roast beef and turkey meat slices, despite treatments being otherwise identical, suggesting that phages may be immobilized on the meat surface to varying degrees based on the meat type (Chibeu et al., 2013; Shannon et al., 2020). Phages rely on diffusion to reach their hosts (Wilkinson, 2001), so immobilization on a solid, complex matrix like poultry could reduce efficacy of phages for biocontrol in these products. While this could be minimized by using a higher concentration of phages to increase the odds of reaching a target cell (Moye et al., 2018), it may be possible that using an added surfactant could help phage mobility across the surface.

The efficacy of phage can be also be increased or hindered by additives to the food. Some disinfectants, such as ethanol and peracetic acid, used in the food processing environment can inactivate phages (Kajiura et al., 2001; Moye et al., 2018; Quiberoni et al., 1999; Tomat et al., 2014). In contrast, several food additives in combination with phages have helped to achieve greater reductions in pathogen numbers. In particular, levulinic acid

(Magnone et al., 2013), nisin (C. Wang et al., 2017), potassium sorbate (C. Wang et al., 2017), potassium lactate, and sodium diacetate (Chibeu et al., 2013; Shannon et al., 2020) are reported to work well in combination with phages in food applications. Lauric arginate, a cationic surfactant, has also proven effective in working synergistically with *Salmonella* phages to reduce the pathogen levels in chicken products (Sukumaran et al., 2015). Lauric arginate is known for antimicrobial activity as a food additive, but it is possible that its surfactant properties aided the phage mobility. In this study, a six-phage cocktail was applied to chicken samples inoculated with *Salmonella* Typhimurium to assess the phage efficacy in a food challenge model. Additionally, of 0.1% w/v Tween 80, also known as polysorbate 80 – a GRAS food additive known to be a nonionic surfactant, was applied in combination with the phage cocktail to determine if this would further reduce *Salmonella* levels in the chicken samples by increasing the phages' motility, helping the phages to encounter a host.

4.2 Materials and Methods

4.2.1 Strain and culture conditions

The challenge strain used in this study, *S.* Typhimurium 4/74, and each host strain used for phage propagation and enumeration (Table 1) were cultured at 37ºC for 16-18 hours on Luria-Bertani (LB) broth or LB agar plates.

4.2.2 Phage propagation and cocktail preparation

Phages were propagated the double agar overlay assay. Briefly, 0.1mL of phage lysate and 0.1mL of a culture of propagation strain(s) were mixed in with 4mL of molten 0.35% w/v LB agar. This mixture was poured onto a 1.5% w/v LB agar plate and allowed to solidify. After incubation at 37ºC for 16-18 hours, the top agar layer was mixed with 6mL of SM buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 8mM $MgSO_4$) in a conical tube and centrifuged at 5000rpm for 15 minutes. The supernatant was filter-sterilized through a 0.22um pore size syringe filter (Millipore). Phage titers were measured by serially diluting the lysates, plating each dilution in a double agar overlay on their host strain(s), and incubation for 16-18 hours overnight to determine the number of plaque-forming units (PFU) per mL. To prepare the phage cocktail, each phage was diluted in phosphate buffered saline (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) to 10^9

PFU/mL if necessary. One milliliter aliquots of each phage at 10^9 PFU/mL were combined into a conical tube, and the cocktail was stored at 4ºC until use.

Table 4.1. Challenge strain and phages used in this study

Challenge strain		
S. Typhimurium 4/74		
Phage	**Host(s)**	
	Strain	*Source*
EH1	*S.* Typhimurium LT2 SGSC 1412	SGSC
EH2	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC
EH3	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Enteritidis SGSC 2475	SGSC
	S. Reading E2018018984	MDH
	S. Infantis I2018008804-1	MDH
EH4	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC
EH5	*S.* Typhimurium LT2 SGSC 1412	SGSC
	S. Typhimurium LT2 SGSC 288 – *waaL* mutant	SGSC
EH6	*S.* Typhimurium 4/74	Jay Hinton
	S. Kentucky PDX AB7	PDX
	S. Montevideo ATCC 8387	PDX

4.2.3 Chicken breast application

Frozen, raw chicken breasts were purchased from a local supermarket and stored at -20ºC until use. Chicken breasts were thawed for 48 hours at 4ºC, then aseptically sliced into 25 (±0.5) gram pieces. Chicken samples were placed into individual stomacher bags with filters (Nasco Whirl-Pak) and transferred into a biosafety cabinet. Chicken samples were inoculated by pipetting 0.1mL of a culture of *S.* Typhimurium 4/74 diluted in PBS to achieve an inoculation level of 10^5 CFU/g. Sterile spreaders were used to distribute cells evenly across the chicken surface, then samples were dried for 15 minutes at room temperature in the biosafety cabinet. Non-inoculated chicken samples were included as a negative control. Inoculated samples received 0.1mL of phage cocktail to achieve a multiplicity of infection (MOI) of 100, 0.1% w/w Tween 80 alone, or both 0.1mL of phage cocktail and 0.1% w/w Tween 80, or PBS for the no treatment group. Each treatment was spread with sterile spreaders to distribute across the sample surface, and samples were dried for 15 minutes after treatment application. The samples were sealed and stored for 14 days at 4ºC. Cell counts were performed after 14 days of incubation by adding 225mL of PBS to each stomacher bag and homogenizing samples in a stomacher for 60 seconds each. Each sample was plated in triplicate by spreading 0.1mL on XLT4 agar plates. Plates were incubated for 24 hours at 37ºC prior to enumeration of cells.

4.2.4 Statistical analyses

Unpaired, one-tailed T-tests were performed in Excel to determine if any of the treatment groups – 0.1% w/w Tween 80, the phage cocktail, or both 0.1% w/w Tween 80 and the cocktail combined – had significantly different *Salmonella* levels from the no treatment group. A p-value less than 0.05 was considered statistically significant.

An unpaired, two-tailed T test was performed in Excel to determine if there was a significant difference in *Salmonella* levels between the phage cocktail treatment and the phage cocktail plus 0.1% w/w Tween 80 treatment.

4.3 Results and Discussion

The phage cocktail alone reduced the level of *Salmonella* on the chicken breast samples significantly ($p < 0.05$), a nearly 0.7 log reduction (Table 4.2; Table 4.3). This is similar to results seen in other *Salmonella* phage studies in food models where reductions have ranged from 0.4 log to 3 logs were reported (Moye et al., 2018). The MOI used in this

study was approximately 100 phages per every 1 *Salmonella* cell, or an MOI of 100. MOI is an important determinant of phage efficacy (Berchieri et al., 1991; Greer, 1988; Guenther et al., 2009; Hagens & Loessner, 2010; Soffer et al., 2017) because a greater density of phage particles in a given area or volume compared to host cells increases the chances that a phage will encounter a host. Approximately 10^7 PFU/g was applied to the chicken, although some recommendations state that no fewer than 10^8 PFU/g should be used (Guenther & Loessner, 2011). Thus, increasing the MOI and phage density may result in greater reductions of *Salmonella* by this cocktail. Additionally, the food matrix dictates the efficacy of the phages in part, meaning that this cocktail may prove more or less effective for other food products. It may be concluded, though, that the cocktail has the capacity to supplement other measures for reducing *Salmonella* in poultry and other products.

The addition of 0.1% w/w Tween 80 to the chicken did also significantly reduce the *Salmonella* levels when applied in combination with the phage cocktail (Table 4.3). No reduction was seen with the addition of 0.1% w/w Tween 80 alone, indicating that it did not have an antimicrobial effect. The phage cocktail with the addition of the surfactant yielded a nearly 0.5 log reduction, which is roughly 15% less than the phage cocktail alone (Table 4.2). Reductions in Salmonella cell counts for each treatment group in comparison to the no treatment group can be seen in Figure 4.1.

The difference in the remaining *Salmonella* counts using phage alone versus phage with the addition of the surfactant was statistically significant (data not shown). It appears that while a fair reduction occurred in the presence of 0.1% w/w Tween 80 with the phage cocktail, the surfactant may have hindered the phage rather than aiding its mobility. Because phages are charged, with the heads usually possessing a negative charge and the tails possessing a positive charge (Rosner & Clark, 2021), 0.1% w/w Tween 80 was selected due to its nonionic nature, in addition to being GRAS. The aim of this was to avoid major interactions between the phage and a charged surfactant, and nonionic surfactants have proved helpful in using phages to control biofilms in healthcare settings (Santiago et al., 2020). However, a study evaluating the impacts on nonionic, ionic, and biological surfactants on phage survival and ability to adsorb to sorbents found that

surfactants reduced phage survival and prevented phages from adsorbing by either displacing the phages or by occupying available positions on the sorbent. Interestingly, the authors conclude that reduction in sorption may allow for greater phage mobility and that nonionic surfactants are less likely to harm phages than ionic surfactants (Devamita Chattopadhyay et al., 2002). The interactions between surfactants, proteins, lipid membranes, and structures is incredibly complex (Heerklotz, 2008; Otzen, 2011), leaving many questions about the interactions between Tween 80, phages, and the molecular structures involved in this model that are beyond the scope of this study. Overall, it seems that there may be a detrimental effect of Tween 80 on phages, and this may be a necessary consideration when deciding product formulation if phages are going to be added.

Table 4.2. Mean *S.* Typhimurium strain 4/74 cells recovered from chicken pieces after 14 days.

Treatment	**CFU per 25g (± SD*)**	**Log CFU per 25g (± SD*)**
No Treatment	$8.89 \cdot 10^5$ ($\pm$ $2.43 \cdot 10^4$)	5.94 ($\pm$ 0.12)
0.1% Tween 80 Only	$1.02 \cdot 10^6$ ($\pm$ $4.48 \cdot 10^4$)	5.97 ($\pm$ 0.17)
Phages + 0.1% Tween 80	$3.09 \cdot 10^5$ ($\pm$ $4.80 \cdot 10^5$)	5.49 ($\pm$ 0.07)
Phages Only	$1.90 \cdot 10^5$ ($\pm$ $2.35 \cdot 10^5$)	5.28 ($\pm$ 0.05)

*SD – standard deviation. Means were calculated from 3 biological replicates with 3 technical replicates for each condition.

Table 4.3. Reduction in *S.* Typhimurium strain 4/74 cell recovery for each treatment group compared to the no treatment group ($p < 0.05$, n=3).

Condition	**Log Reduction**	**% Reduction**	**Significantly Different**
0.1% Tween 80 Only	-0.06	-15%	No
Phages + 0.1% Tween 80	0.46	65%	Yes
Phages Only	0.67	79%	Yes

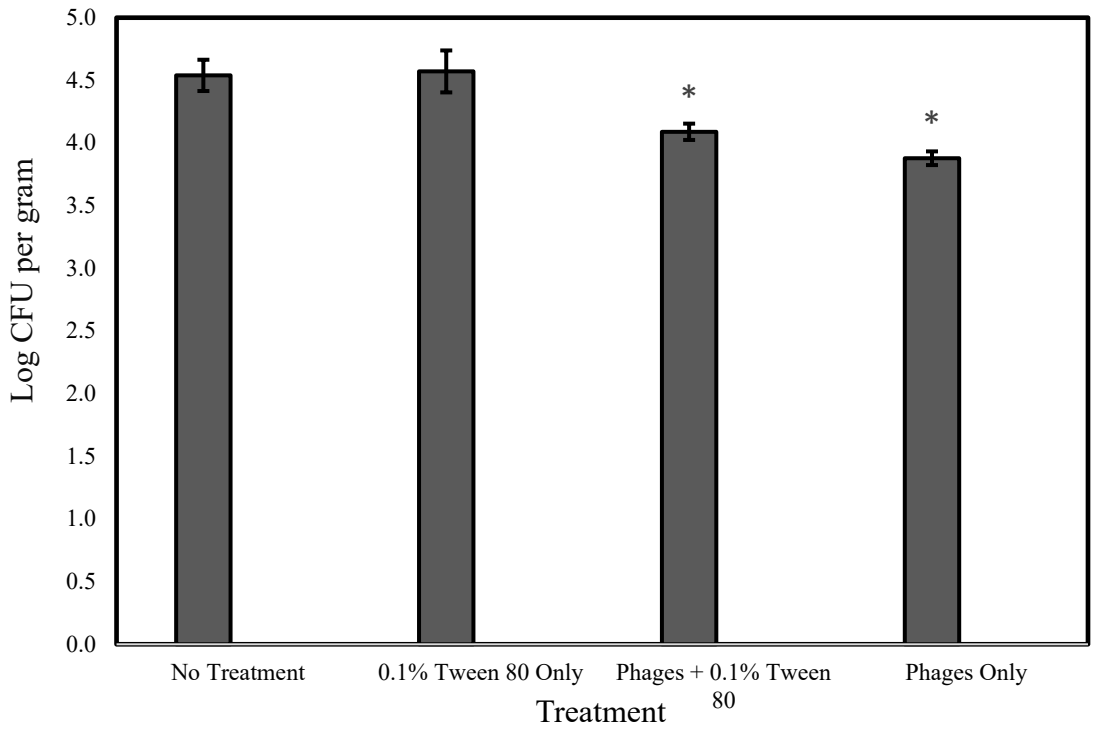


*Figure 4.1. Reduction in S. Typhimurium strain 4/74 cell recovery for each treatment group compared to the no treatment group. * indicates a significant difference from no treatment group ($p < 0.05$, $n=3$). Bars represent the means of 3 biological replicates, consisting of 3 technical replicates each.*

4.4 Conclusion

Chicken and other poultry remain major vehicles for *Salmonella* outbreaks. Bacteriophage cocktails have been proposed for biocontrol of *Salmonella* in food systems, including in raw poultry. This proposed phage cocktail significantly decreased the level of *S.* Typhimurium 4/74 in chicken breasts by nearly 0.7 log when applied at an approximate MOI of 100. The surfactant Tween 80 did not act synergistically with the phage cocktail to reduce *Salmonella* levels in the chicken but rather appeared to have an antagonistic effect. This may be necessary to take into consideration when applying phage cocktails for biocontrol in products formulated with Tween 80. Overall, this putative phage cocktail shows promise for reducing *Salmonella* in food products. It may be particularly useful in a hurdle approach i.e., in combination with other food preservation methods. Further testing on other food matrices will give a more comprehensive view of the abilities of this cocktail as a biocontrol agent.

Chapter 5: Conclusions

Bacteriophages are promising new biocontrol agents for pathogens in the food system as an alternative to traditional processing techniques and chemical antimicrobials. The specificity, safety, and preservation of the organoleptic properties of foods make phages especially ideal for raw and ready-to-eat foods which do not undergo any kill-step. Because phages are so numerous, there is a never-ending wealth of phages to be discovered for use as biocontrol agents. In this study, wastewater samples from local facilities were used to isolate 27 unique bacteriophages capable of infecting *Salmonella*. The host ranges were assessed for the phages on 19 strains of *Salmonella*, and a six-phage cocktail was produced based on their complimentary host ranges. The cocktail was also effective against four STEC serotypes. The receptors for the phages were determined to be BtuB, a porin that aids in vitamin B12 import into the host cell. This is a common receptor, but it gave no insights into the varied host ranges of the phages. In a chicken model, the cocktail reduced *Salmonella* levels by approximately 0.7 logs, indicating nearly 80% of *Salmonella* cells applied to the chicken were lysed. The addition of a nonionic surfactant, Tween 80 to the chicken surface prior to phage application reduced the efficacy of the cocktail. This may be important to consider when applying phage cocktails to a food product formulated with Tween 80. To summarize, a putative phage cocktail was identified which shows promise as a potential biocontrol agent for both *Salmonella* and STEC in food applications.

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Appendix

Table A1. Sensitivity of *Salmonella* and STEC strains to individual isolated phages

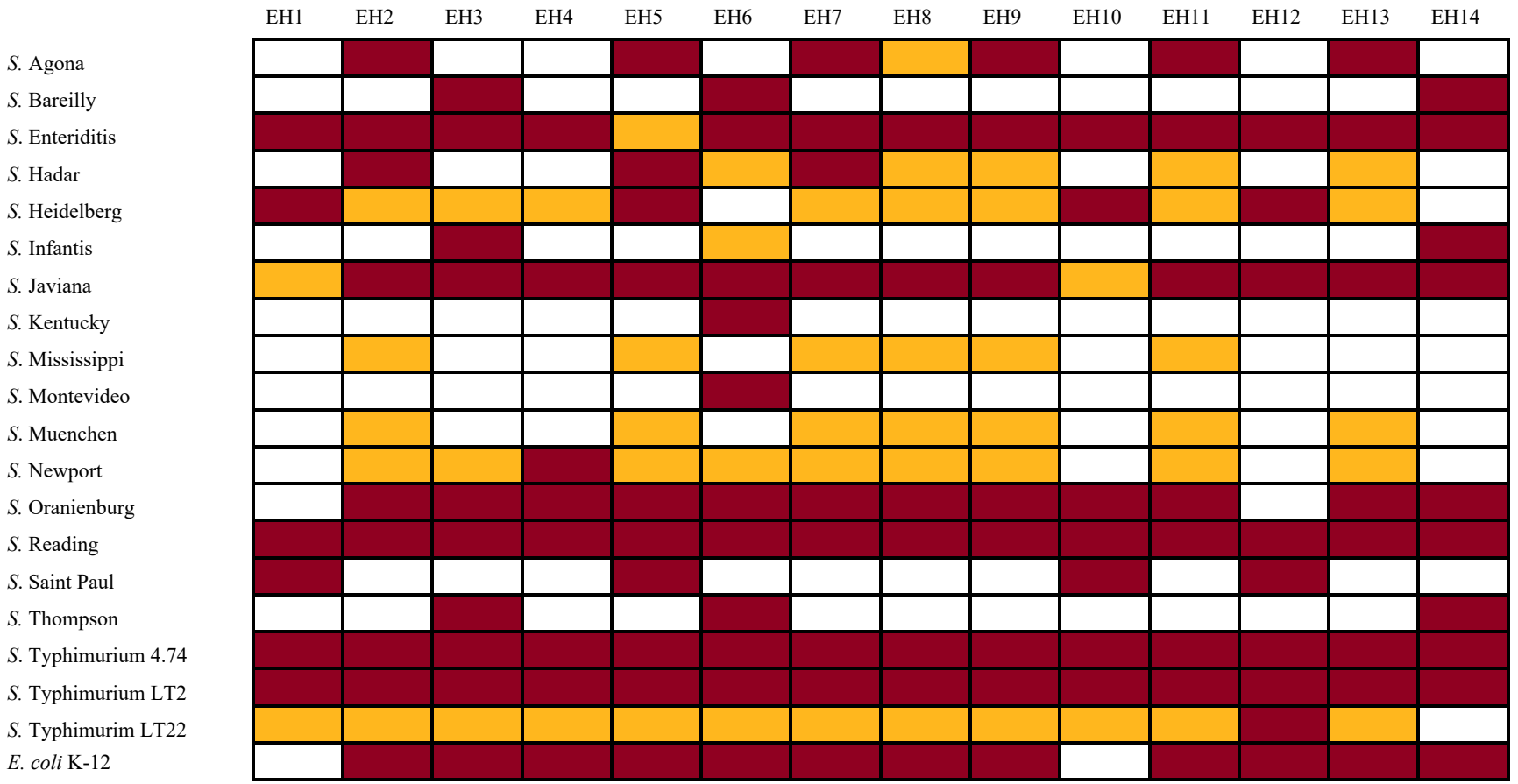

Table A1 Continued.

	EH15	EH16	EH17	EH18	EH19	EH20	EH21	EH22	EH23	EH24	EH25	EH26	EH27
S. Agona													
S. Bareilly													
S. Enteriditis													
S. Hadar													
S. Heidelberg													
S. Infantis													
S. Javiana													
S. Kentucky													
S. Mississippi													
S. Montevideo													
S. Muenchen													
S. Newport													
S. Oranienburg													
S. Reading													
S. Saint Paul													
S. Thompson													
S. Typhimurium 4.74													
S. Typhimurium LT2													
S. Typhimurim LT22													
E. coli K-12													

Sensitive Partially sensitive Resistant